A RECEPTOR'S POINT OF VIEW

Studying interactions between TGF-β and IL-6 signaling in articular cartilage

Renske Wiegertjes

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Studying interactions between TGF-β and IL-6 signaling in articular cartilage

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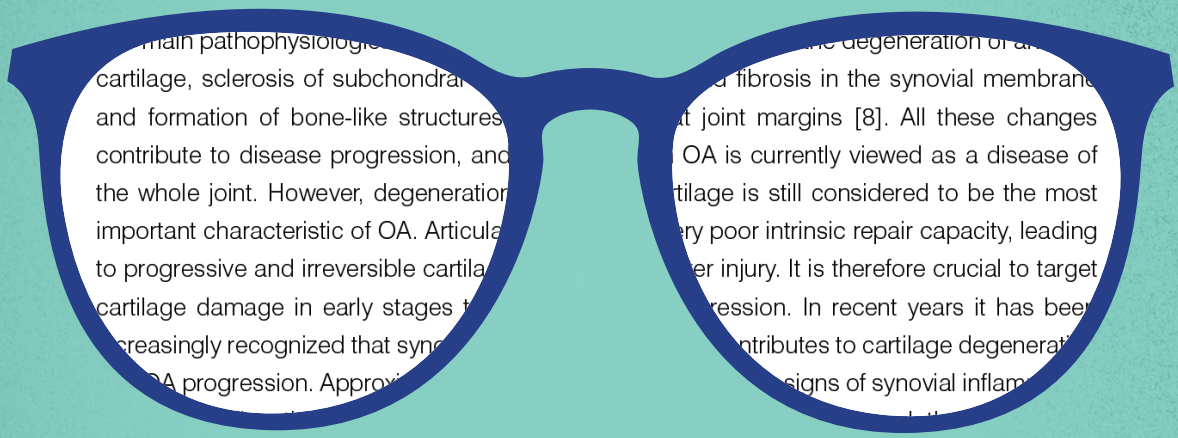
TABLE OF CONTENTS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Chapter 1

General introduction and outline of this thesis

OSTEOARTHRITIS

Osteoarthritis (OA) is a degenerative joint disease and is the most prevalent joint disorder with increasing impact worldwide, currently affecting approximately 300 million people globally [1, 2]. Several risk factors for OA have been identified, including ageing, obesity, gender, genetic predisposition and joint trauma [3]. However, OA pathophysiology is multifactorial and its etiology remains unknown. Due to the expected rise in life expectancy and body weight in western countries, the incidence of OA is predicted to keep rising in coming years putting a large strain on health care and costs [1, 3]. OA can affect any joint, but is mostly observed in the knee, hands, hip and spine, causing symptoms such as severe pain, stiffness and loss of joint function [4, 5]. It is already regarded as one of the ten most disabling diseases in developed countries and symptoms are often accompanied with severe functional impairment [6]. Unfortunately, current treatment options do not modify disease outcome, and are mainly focused on life-style adjustments, pain relief, or joint-replacement surgery at end-stage of the disease [1, 7].

The main pathophysiological hallmarks of OA are considered to be the degeneration of articular cartilage, sclerosis of subchondral bone, inflammation and fibrosis in the synovial membrane and formation of bone-like structures (osteophytes) at joint margins [8]. All these changes contribute to disease progression, and for this reason OA is currently viewed as a disease of the whole joint. However, degeneration of articular cartilage is still considered to be the most important characteristic of OA. Articular cartilage has very poor intrinsic repair capacity, leading to progressive and irreversible cartilage degeneration after injury. It is therefore crucial to target cartilage damage in early stages to prevent further progression. In recent years it has been increasingly recognized that synovial inflammation actively contributes to cartilage degeneration and OA progression. Approximately 50% of the OA patients show signs of synovial inflammation and this significantly correlates with cartilage damage and pain [9-14]. Although the actual cause of synovitis in OA is unknown, cells in the synovium can be triggered by endogenous danger signals derived from damaged tissue, such as cartilage matrix fragments or alarmins [15-18]. In response, the OA synovium produces several inflammatory mediators, such as pro-inflammatory cytokines, alarmins and chemokines, which directly contribute to the initiation and progression of articular cartilage degeneration [9, 19, 20]. The interaction between local cartilage damage and the synovium leads to a state of low-grade, chronic joint inflammation, and ultimately results in disease progression and clinical OA development [21, 22]. An improved understanding of how inflammation contributes to the onset and progression of cartilage damage in OA could therefore enable the development of future targeted therapies.

CHANGES IN ARTICULAR CARTILAGE

Articular cartilage is a highly specialized connective tissue which facilitates joint function by providing a smooth, lubricated surface and reducing the frictional coefficient [23]. The unique structural organization of the cartilage matrix is crucial for its biomechanical properties. Chondrocytes are responsible for maintenance of the extracellular matrix (ECM), which contains a complex network of collagen type II and proteoglycans such as aggrecan [24, 25]. Collagen type II proteins provide structure and strength to articular cartilage, while water-attracting proteoglycans serve as lubricants that help withstand high compressive forces. To maintain a healthy cartilage ECM, chondrocytes regulate essential anabolic processes like the production of ECM components and the ECM-cross linking enzymes that strengthen the matrix structure [23, 26]. On the other hand, they also control catabolic processes, such as the production of matrix degrading enzymes, which mediate healthy remodeling of the cartilage matrix.

However, during OA, the delicate balance between anabolic and catabolic processes shifts towards catabolism, resulting in breakdown of the cartilage matrix (schematic overview in Fig. 1). Increased production and activation of cartilage degrading enzymes like matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) have been recognized as a key event in the initiation and progression of cartilage degeneration in OA [27-29]. Of the MMP and ADAMTS families, especially MMP-3, MMP-9 and MMP-13 have been implicated in collagen degradation, and ADAMTS-4, ADAMTS-5, MMP-3 and MMP-13 in aggrecan breakdown [30, 31]. Remarkably, also anabolic activities of chondrocytes are increased in the initial phase of OA, indicated by chondrocyte proliferation clusters and increased proteoglycan production [27, 29, 32]. However, this is not sufficient to restore the ECM balance, and could be regarded as an inadequate attempt to maintain cartilage integrity [29]. Moreover, at this stage chondrocyte clusters are observed and chondrocytes differentiate towards a hypertrophic-like state which resembles the phenotype of growth plate chondrocytes. Hypertrophic chondrocytes are characterized by expression of hypertrophic markers such as MMP-13 and Collagen type X, and contribute to matrix instability and degeneration [33, 34]. Altogether, this shows that chondrocyte homeostasis is severely disturbed in OA and may lie at the root of OA-related cartilage degeneration.

Although many factors control chondrocyte function, two groups of signaling molecules are recognized as key regulators of important anabolic and catabolic processes. First, growth factors have been identified as important regulators of several essential chondrocyte functions like metabolism, differentiation, proliferation, and ECM production and degradation [35, 36]. The transforming growth factor-β (TGF-β) family is an especially important group of growth factors in cartilage, as it controls chondrocyte homeostasis on all these levels and has important anti-inflammatory and anti-hypertrophic properties [37, 38]. Secondly, pro-inflammatory cytokines

have been identified as important regulators of mainly catabolic processes in cartilage. For example, they stimulate chondrocytes to produce and activate matrix degrading enzymes or inhibit synthesis of ECM molecules thereby contributing to cartilage degeneration [39-47]. The pro-inflammatory cytokine interleukin-6 (IL-6) is highly increased in OA synovial fluid and directly modulates chondrocyte function e.g. via induction of matrix-degrading enzymes [42, 48]. However, the role of IL-6 in cartilage and OA development has not received much attention so far. It is evident that TGF-β and IL-6 both play important roles in regulating cartilage homeostasis and degeneration, but a link between these two factors in cartilage remains to be identified. In this thesis, we focus on interactions between the protective growth factor TGF-β and the detrimental cytokine IL-6 in chondrocytes. Therefore, we first elaborate on the role of TGF-β signaling in cartilage homeostasis, and thereafter discuss the contribution of IL-6 to the development of cartilage degeneration and OA.

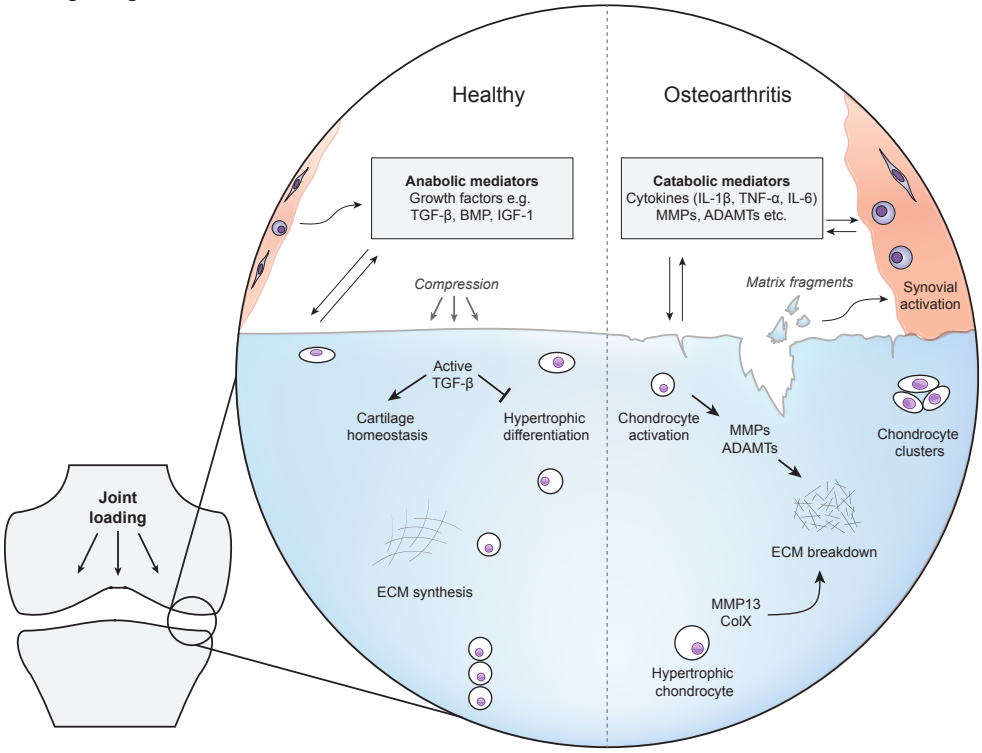

Figure 1. Cartilage degeneration in osteoarthritis. Schematic overview of healthy cartilage and synovium (left). Anabolic mediators, such as TGF-β, maintain cartilage homeostasis by repressing hypertrophic differentiation and regulating matrix synthesis. On the right, osteoarthritic cartilage and synovium is depicted which shows the main pathological changes of the disease. The balance between anabolic and catabolic processes shifts towards catabolic breakdown of the cartilage matrix by degenerative mediators such as MMPs. The synovial membrane is activated and releases pro-inflammatory cytokines and matrix degrading enzymes. Moreover, altered TGF-β signaling results in hypertrophic differentiation of articular chondrocytes. TGF-β: transforming growth factor-β, MMPs: matrix metalloproteinases.

TGF-β SIGNALING IN HEALTHY AND AGEING CARTILAGE

The growth factor TGF-β is essential for the formation and maintenance of healthy articular cartilage [49, 50]. It has a central role in cartilage homeostasis, and disruption of TGF-β signaling has been implicated in OA development. TGF-β signaling induces receptor-mediated phosphorylation of intracellular mediators called Smad proteins, which translocate to the nucleus and activate target gene expression [51]. TGF-β has the ability to induce two intracellular signaling pathways leading to Smad2/3 or Smad1/5/9 activation. Which pathway is activated is determined by TGF-β concentration, TGF-β receptor expression, and the expression of co-receptors [52, 53]. Lower concentrations of TGF-β mainly activate Smad2/3 signaling, while activation of Smad/1/5/9 signaling is increased at higher concentrations [54, 55]. Moreover, activation of the TGF-β type I receptor ALK5 stimulates Smad2/3 signaling in cartilage, while activation of ALK1 promotes the Smad1/5/9 signaling route [55, 56]. On a functional level, both pathways have profoundly different effects and even have been shown to antagonize each other [53, 57]. Signaling via the Smad2/3 route protects cartilage against the development of chondrocyte hypertrophy and blocks the production of catabolic enzymes [37, 52]. Indeed, mice with loss of Smad3 function develop degenerative OA-like joint disease, with progressive cartilage damage, osteophyte formation and increased expression of collagen type X [58]. In contrast, signaling via the Smad1/5/9 route promotes chondrocyte hypertrophic differentiation, and is associated with MMP-13 expression [53, 59, 60]. Besides its role in regulating chondrocyte hypertrophy, TGF-β controls matrix maintenance and production. On one hand, TGF-β regulates the synthesis of matrix components like proteoglycans and collagen type II [61-63]. On the other hand TGF-β blocks the expression of catabolic mediators, for instance by inducing metalloproteinase inhibitor expression thereby preventing matrix degeneration [64, 65]. TGF-β also has an important anti-inflammatory function in cartilage, as it can actively block the effects of pro-inflammatory cytokines [52]. However, during ageing or osteoarthritis TGF-β signaling is altered which contributes to disrupted cartilage homeostasis.

Ageing is strongly associated with OA development, and changes in ageing articular cartilage have been extensively investigated [66]. Ageing affects TGF-β signaling in multiple ways, which has been postulated to be a potential cause for age-related OA development. Our lab has previously shown that TGF-β signaling via Smad3 is decreased in murine and bovine cartilage with age, which was associated with increased cartilage degeneration and reduced repair capacity [67-70]. Moreover, expression of TGF-β itself, as well as chondrocyte responsiveness towards TGF-β decreases markedly with age [68, 71]. Possibly, this reduction in TGF-β/Smad3 signaling is caused by age-related changes in TGF-β receptor expression. Our lab has demonstrated that ALK5 expression decreases in ageing bovine cartilage [68], and that there is a shift towards ALK1 signaling in ageing mice which correlates with OA development [53]. Also in OA, TGF-β signaling in cartilage is altered in several ways [38, 72]. First, levels of active TGF-β are increased in OA

due to inflammation, cartilage degradation and high levels of proteases [38, 73, 74]. Secondly, TGF-β signaling shifts from protective p-Smad2/3 signaling towards detrimental p-Smad1/5/9 signaling in chondrocytes [53, 72]. This can potentially be explained by the higher TGF-β levels and/or by increased expression of ALK1 versus ALK5 in OA cartilage [53]. Finally, the increased presence of inflammatory cytokines in OA contributes to altered TGF-β signaling via inhibition of protective Smad2/3 signaling [75, 76]. A better understanding of why TGF-β signaling is altered in ageing or osteoarthritic cartilage, e.g. by investigating the interplay between inflammation and TGF-β signaling in chondrocytes, will help to separate protective and detrimental effects of TGF-β. This will ultimately lead to the development of therapeutic approaches that halt or prevent cartilage damage in OA.

INTERLEUKIN-6 IN OA

The cytokine IL-6 is a multifunctional cytokine with broad-ranging effects in tissue homeostasis and defense by regulating central processes like hematopoiesis and the acute phase response [77]. Increased expression of IL-6 has been implicated in the pathophysiology of several diseases, such as rheumatoid arthritis and Castleman's disease [77, 78], and might also play a role in OA. The IL-6 signaling pathway is activated by binding of IL-6 to the membrane-bound IL-6 receptor (IL-6R). Hereafter, the IL-6/IL-6R forms a complex with the signal transducer glycoprotein 130 (gp130), which results in activation of the Janus kinases/signal transducers and activators of transcription (JAK/STAT) pathway [79, 80]. The regulatory protein suppressor of cytokine signaling 3 (SOCS3) has been identified as a primary inhibitor of the IL-6 pathway, and is essential to control the duration and biological response of IL-6 signaling [81, 82]. SOCS3 restricts IL-6 signaling by binding to gp130 and inhibiting JAK-kinase activity. The IL-6R itself has no signal transduction capacity and is differentially expressed in various tissues. Chondrocytes generally have low IL-6R expression [83, 84], while IL-6R expression is high in certain cell types like monocytes, hepatocytes, and particular leukocyte subsets [85]. Chondrocyte sensitivity to IL-6 may be enhanced during OA, due to changes in mIL-6R expression levels by hormones cytokines and epigenetic factors [85]. In contrast to the IL-6R, gp130 is ubiquitously expressed throughout the body [85, 86]. Interestingly, a soluble variant of IL-6R (sIL-6R) exists, which forms a complex with IL-6 and can then bind to any gp130-expressing cell. This process is termed trans signaling and greatly broadens the scope of IL-6 responsiveness [87, 88]. sIL-6R is produced by shedding of membrane-bound IL-6R receptor or alternative splicing [89, 90]. Signaling via membrane-anchored IL-6R (mIL-6R) is termed classic signaling and has been linked to homeostatic processes (schematic overview in Fig. 2). In contrast, IL-6 trans-signaling mainly regulates pro-inflammatory events and is implicated in numerous chronic diseases and cancers [87, 88].

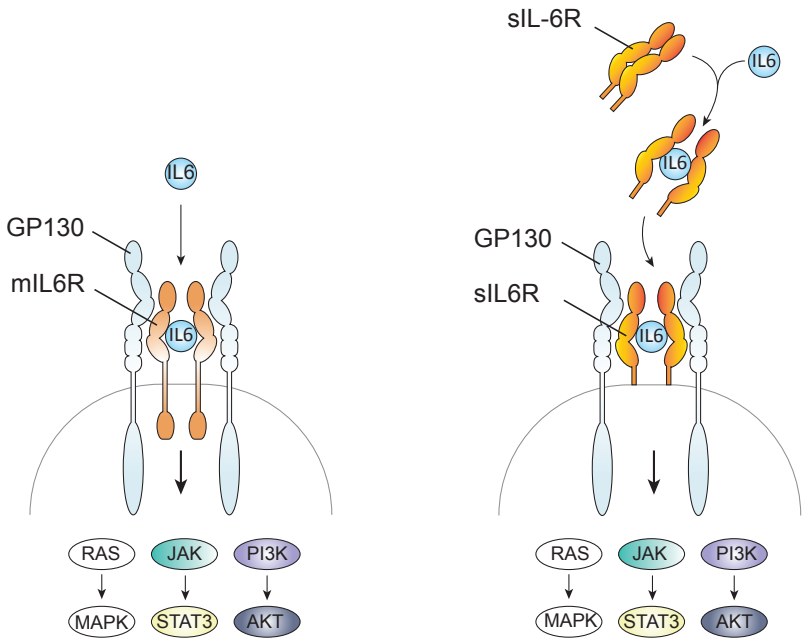

Figure 2. A schematic overview of the IL-6 signaling routes. Classic IL-6 signaling involves cells expressing both membrane (m)IL-6R and gp130; free IL-6 binds to mIL-6R, forming a complex with gp130. IL-6 trans-signaling is activated by pre-formed complexes of IL-6 and soluble IL-6R (IL-6/sIL-6R) and requires only gp130 expression on target cells. After IL-6 binds to the IL-6R, complex formation with gp130 initiates phosphorylation of JAKs resulting in activation of STAT3-, PI3K- and Ras-Raf-MEK-ERK signaling. gp130: glycoprotein 130; IL-6: interleukin-6; IL-6R: IL-6 receptor. JAK: janus kinase; MAPK: mitogen-activated protein kinase; PI3K: phosphoinositide 3-kinase.

Levels of IL-6 and sIL-6R are increased in serum and synovial fluid of OA patients compared to healthy individuals [91, 92], and circulating IL-6 levels have been associated with OA development [93, 94]. Moreover, there are several indications that IL-6 directly contributes to cartilage degradation in OA [42, 48, 95]. IL-6 enhances the expression of MMP-3 and -13 in bovine chondrocytes, as well as the expression of ADAMTS-4 and -5 [48]. Moreover, intra-articular injection of IL-6 in murine knee joints results in cartilage damage and increased production of MMP-3 and -13 [42]. In line with this, therapeutically blocking IL-6 or its downstream mediator STAT3 during experimental OA rescues cartilage damage and osteophyte formation [95]. However, also protective effects of IL-6 have been described such as modest stimulation of proteoglycan synthesis and induction of tissue inhibitors of metalloproteinases [96-98]. These discrepancies may be explained by functional differences in IL-6 classic versus trans signaling routes as will be explained in chapter five of this thesis. Altogether, this shows that IL-6 has detrimental effects in cartilage and contributes to cartilage degeneration in OA.

INTERPLAY BETWEEN INFLAMMATION AND TGF-β SIGNALING IN CARTILAGE

As mentioned, TGF-β has important anti-inflammatory effects in cartilage. Especially interactions between TGF-β signaling and the pro-inflammatory cytokines IL-1β and TNF-α have been studied [99-101]. Our lab has previously shown that TGF-β potently counteracts 38% of the genes regulated by pro-inflammatory cytokine IL-1β in chondrocytes [99]. We also showed that intra-articular injection of TGF-β in mice resulted in protection against IL-1β-induced proteoglycan degeneration [101]. Moreover, it has been reported that TGF-β can protect cartilage against TNF-α-induced collagen breakdown in bovine articular cartilage [100]. How this works is not fully elucidated yet, but several mechanisms have been suggested. For example, TGF-β decreases IL-1 receptor (IL-1R1) expression in rabbit chondrocytes thereby limiting IL-1β signaling [102, 103]. Moreover, TGF-β can activate expression of IL-1 receptor antagonist (IL-1RA) in monocytes, leading to inhibition of IL-1β signaling [104]. Additionally, competition between NF-κβ, the intracellular mediator of IL-1β signaling pathway, and SMAD3 for co-transcription factors has been described as a possible mechanism in endothelial cells [105]. This anti-inflammatory function of TGF-β is lost upon ageing, demonstrated by a reduced ability of TGF-β to counteract IL-1β-mediated inhibition of proteoglycan synthesis and IL-1β-induced nitric oxide production [70].

Vice versa, inflammation itself can also affect TGF-β signaling. For example, IL-1β decreases TGF-β receptor II expression on both mRNA and protein level in human articular chondrocytes resulting in inhibition of TGF-β-induced gene expression [75]. Reduction of TGF-β receptor II expression was also observed in mechanically compressed bovine cartilage after stimulated with conditioned medium obtained from *in vitro* cultured OA human synovial biopsies [106]. Moreover, both IL-1β and TNF-α have been shown to disturb TGF-β signaling in chondrocytes by modulating activation of the intracellular mediators Smad2/3, which prevented TGF-β-mediated collagen type II and aggrecan expression [76]. This can possibly be explained by IL-1β-mediated induction of inhibitory Smad7, which ultimately decreases Smad2/3 activation levels and TGF-β downstream gene expression [107]. However, both TNF-α and IL-1β have been described to reduce SMAD3/4 DNA-binding activity in human OA chondrocytes, independent of SMAD7 [76].

CROSSTALK BETWEEN THE TGF-β AND IL-6 SIGNALING PATHWAYS

Remarkably, interactions between TGF-β and IL-6 signaling have not been studied so far in cartilage. In other tissues, several mechanisms of interaction between the TGF-β and IL-6 signaling pathway have been reported [108-111], however, it differs per cell type whether TGF-β and IL-6 show synergistic or antagonistic effects. In T-cells, for example, TGF-β synergizes with

IL-6 by promoting the degradation of FOXP3 to modulate the activity of regulatory T-cells [109]. Furthermore, IL-6 and TGF-β synergize to stimulate Th17 differentiation via several mechanisms [112]. The combination of TGF-β and IL-6 stimulates the expression of transcription factors RORγt and RORα which mediate differentiation of the Th17 cell lineage [113, 114]. Moreover, TGF-β suppresses expression of SOCS3 in T-cells, resulting in enhanced STAT3 activation which is required for Th17 differentiation [110]. In contrast, in intestinal epithelial cells TGF-β was shown to suppress IL-6 signaling via Smad2-mediated inhibition of STAT1 and STAT3 phosphorylation which may be protective in the context of inflammatory bowel disease [108]. Also in macrophages, antagonism between the IL-6 pathway and TGF-β pathway has been described [115]. Here it was shown that the increased SOCS3 expression in macrophages antagonizes TGF-β signaling via direct inhibition of Smad3 [115]. Also during joint inflammation, SOCS3 is highly expressed in both the synovium and articular cartilage [116]. As TGF-β signaling is crucial for cartilage homeostasis, increased SOCS3 expression in articular cartilage expression may lead to disturbed TGF-β signaling as observed in OA. However, interactions between SOCS3 and TGF-β signaling have not yet been investigated in articular cartilage. Crosstalk between SOCS3 as negative regulator of the IL-6 signaling pathway, and TGF-β signaling has been reported in several other studies. Loss of SOCS3 in T-helper cells results in hyperproduction of TGF-β [117], and loss of SOCS3 in the liver promotes fibrosis by enhancing TGF-β production [118]. Besides cell type, possibly also the (patho)physiological context determines the outcome of TGF-β and IL-6 crosstalk, such as during tumorigenesis. Signaling molecules Smad3 and STAT3 can engage in direct physical interaction [119-122], which has antagonistic effects during the early phase of tumorigenesis but synergistic effects during late-phase progression [123]. Altogether, these studies indicate potential crosstalk between the TGF-β and IL-6 signaling pathway, which is not yet explored in chondrocytes. Because of the detrimental role of IL-6 in cartilage degeneration [42, 48, 95] and the protective role of TGF-β [37, 52, 69] in cartilage homeostasis, potential interplay between these pathways may have important implications for cartilage homeostasis and OA development.

AIM AND OUTLINE OF THIS THESIS

In this thesis, we investigated the interplay between components of the IL-6 and TGF-β signaling pathway in articular cartilage and explored implications thereof in ageing and OA. In the first two chapters (chapter 2 and 3) we studied two potential mechanisms of interplay between the IL-6 and TGF-β signaling pathway, as illustrated in Figure 3. In the chapters thereafter (chapter 4 and 5), we explore the implications of our findings in the context of ageing and OA.

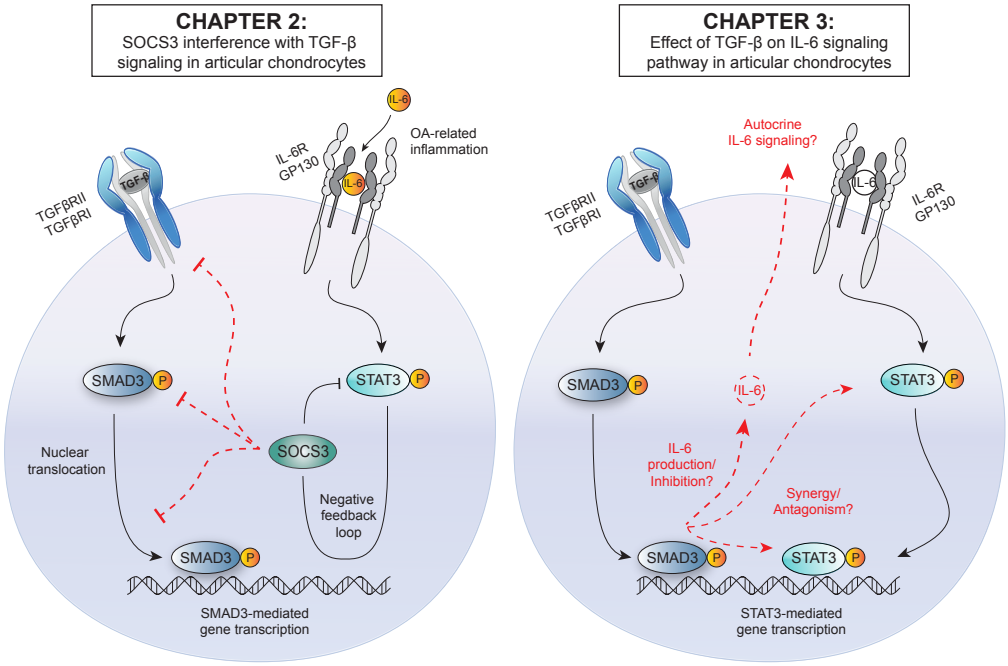

Figure 3. Schematic representation of potential interplay (indicated in red) between the TGF-β and IL-6 signaling pathways as studied in chapter 2 and 3 of this thesis.

First, in **chapter two** we investigated whether SOCS3, as important regulator of the IL-6 signaling pathway, interferes with protective TGF-β/Smad3 signaling in articular cartilage. To investigate this, we studied the effect of adenoviral overexpression of SOCS3 on different components of the TGF-β signaling pathway in human articular chondrocytes. To reach high expression levels, a human chondrocyte cell line (G6 chondrocytes) was used for adenoviral studies. We investigated the effect of SOCS3 on TGF-β receptor expression, as well as functional TGF-β signaling such as TGF-β-induced transcriptional activity, p-Smad activation, and activation of target gene expression. As OA-related inflammation can strongly impair TGF-β-dependent cartilage repair, we additionally investigated whether SOCS3 interferes with TGF-β-mediated cartilage formation. To study cartilage formation, we used bone-marrow derived MSCs transduced with a SOCS3 adenovirus cultured in a three-dimensional pellet culture model. Cartilage formation was determined in the pellets by histological analysis and quantification of proteoglycan content. Based on our findings, we discuss whether therapeutic targeting of SOCS3 in inflammatory OA could help prevent disrupted TGF-β signaling in chondrocytes or help improve TGF-β-dependent cartilage repair processes.

Regulation of IL-6 signaling by TGF-β has been reported in various cell types [108-111]. However, it remains unknown if TGF-β also modulates IL-6 signaling in cartilage, which could

be important to control detrimental IL-6 effects. Therefore, we investigated in **chapter three** whether TGF-β regulates IL-6 signaling in articular chondrocytes. To be able to study different IL-6 signaling pathway components in a standardized manner a human chondrocyte cell line (G6 chondrocytes) was used. In addition, main findings were verified in freshly isolated primary human OA chondrocytes. We first determined if TGF-β regulates IL-6 expression itself and thereby affects autocrine IL-6 signaling via p-STAT3. This was studied in the human G6 chondrocyte cell line, and validated in freshly isolated primary human OA chondrocytes. To investigate if TGF-β also regulates exogenous IL-6 signaling, we performed co-stimulation experiments with TGF-β and recombinant IL-6. Subsequently, we analyzed IL-6-mediated induction of p-STAT3 and target gene expression with and without the presence of TGF-β. To identify potential mechanisms via which TGF-β may regulate IL-6 signaling in chondrocytes, we determined the effect of TGF-β on IL-6R expression in both G6 and primary chondrocytes. Interestingly, we uncovered that TGF-β regulates IL-6R expression in chondrocytes, which is an entirely new concept and has not been reported until now. Via this unique mechanism TGF-β can regulate IL-6 signaling in cartilage, which could be crucial to restrict pro-inflammatory IL-6 effects and preserve cartilage homeostasis.

After we established that TGF-β regulates IL-6 signaling in cartilage, we hypothesized that ageing might affect this regulation due to the altered TGF-β signaling that our lab previously found in ageing cartilage [53, 67, 68, 101]. Therefore, in **chapter four**, we investigated if TGF-β-mediated regulation of IL-6R was indeed lost in aged cartilage, and whether this subsequently results in increased IL-6 signaling. To study this, we used cartilage obtained from bovine metacarpophalangeal (MCP) joints, which can easily be obtained from cows of a wide age-range. Macroscopic selection of healthy bovine cartilage allows for the separation of ageing and OA which is difficult in murine or human cartilage where OA is often concomitant [68]. As many changes develop in the cartilage extracellular matrix with advancing age we used freshly isolated cartilage explants, and not isolated chondrocytes, in this study [124]. As hypothesized, we identified considerable age-related changes in IL-6R expression in flash-frozen bovine articular cartilage. Therefore, we studied whether the response to IL-6 stimulation was different in young versus aged cartilage by analyzing activation of p-STAT3 and OA-related degenerative markers. Finally, we explored whether TGF-β-mediated regulation of IL-6R expression and signaling was lost in bovine cartilage with advancing age. As IL-6 has a detrimental role in cartilage, loss of TGF-β-mediated dampening of IL-6 signaling with advancing age might be important in age-related OA development.

In chapter three and four we established an important role of TGF-β in restricting IL-6 signaling in articular cartilage, which might be lost during ageing or inflammation. This pointed to IL-6 as a potential therapeutic target in OA. Although IL-6 is present in high levels in OA patients and has a pivotal role in OA development, it has not been a primary target of interest so far. In **chapter**

five we review current evidence regarding the role of IL-6 in OA pathophysiology, and discuss potential therapeutic approaches to target the IL-6 signaling pathway in OA. We provide a novel point of view by focusing on the unique ability of IL-6 to signal via a classic- and trans-signaling route. Based on this interesting standpoint, we discuss the opposing effects of these signaling routes in local joint tissues as well as experimental OA mouse models. Moreover, we evaluate currently available IL-6 targeted therapies and discuss whether a better focus on these different IL-6 signaling routes might lead to better therapeutic strategies for future OA treatment.

In chapter **six**, we summarized and discussed the results as described in this thesis, and elaborate on potential implications for cartilage degeneration and OA treatment.

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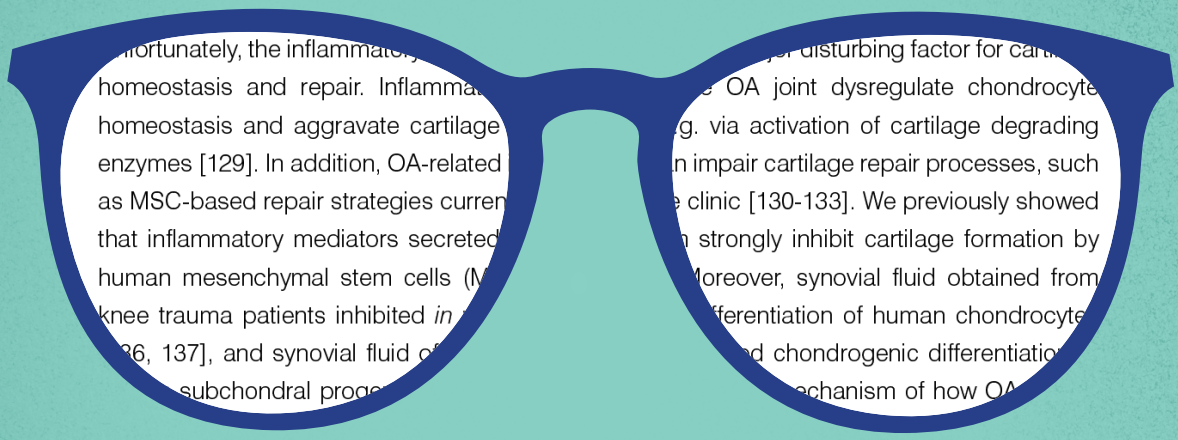
ortunately, the inflammat
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homeostasis and aggravate cartilage
enzymes [129]. In addition, OA-related
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Chapter 2

Inflammation-induced SOCS3 impairs stem-cell based cartilage formation but does not affect protective TGF-β signaling in articular chondrocytes

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In preparation

ABSTRACT

Objective

Inflammation in osteoarthritis (OA) contributes to disrupted cartilage homeostasis, for instance by dysregulating protective TGF-β signaling. Previously, it was shown that the cytokine-inducible protein suppressor of cytokine signaling 3 (SOCS3) inhibits TGF-β signaling in macrophages. Here, we investigated if inflammation-induced SOCS3 is able to disturb TGF-β signaling in articular chondrocytes and/or TGF-β-mediated cartilage formation and thereby could contribute to damage in OA.

Design

The impact of OA-related inflammation (IL-1β, IL-6 or OA synovium-conditioned medium) on TGF-β transcriptional activity and SOCS3 gene and protein expression was determined in articular chondrocyte cell lines (G6, H4). Moreover, increased SOCS3 levels were achieved using adenoviral overexpression in human G6- and bovine chondrocytes, whereafter TGF-β signaling was analyzed by measuring Smad3 transcriptional activity, C-terminal Smad-phosphorylation, and Smad3-dependent gene expression. The effect of SOCS3 on cartilage formation was studied using lentiviral overexpression during a three-dimensional MSC-based culture model to mimic chondrogenesis.

Results

In articular chondrocytes, OA-related inflammatory mediators inhibited TGF-β transcriptional activity, and simultaneously increased SOCS3 expression. However, adenoviral overexpression of SOCS3 itself did not alter TGF-β transcriptional activity, C-terminal activation, nor nuclear translocation of p-Smad3 in articular chondrocytes. Moreover, relative activation of protective ALK5-Smad2/3 versus deleterious ALK1-Smad1/5/9 signaling was not altered by SOCS3 overexpression either. In line with this, increased SOCS3 levels did not affect activation of TGF-β responsive genes like *SERPINE1* and *SMAD7*. Interestingly, overexpression of SOCS3 did strongly impair TGF-β-dependent MSC-based cartilage formation, resulting in reduced pellet sizes and sGAG content.

Conclusions

In contrast to earlier findings in macrophages, our results show that SOCS3 does not alter TGF-β signaling in articular chondrocytes on the level of receptor expression, p-Smad activation or TGF-β transcriptional activity. Strikingly, SOCS3 did strongly impair MSC-based cartilage formation, suggesting that inflammation-induced SOCS3 might impair cartilage repair in OA. However, given our findings in chondrocytes, this effect is most likely independent of SOCS3 directly affecting TGF-β signaling.

INTRODUCTION

Progressive degeneration of articular cartilage is the main characteristic of osteoarthritis (OA). Although cartilage degeneration was considered as a simple wear and tear process in the past, nowadays it is known that the whole joint is involved and that inflammation significantly contributes to OA pathogenesis [125, 126]. Inflammation of the synovial membrane is observed in ~50% of OA patients and several studies showed a positive correlation between synovial inflammation and cartilage damage [9, 10]. The activated synovium releases inflammatory mediators such as interleukin(IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6, which stimulate the production of cartilage matrix degrading enzymes [127, 128]. Increased production of cartilage degrading enzymes results in degeneration of essential cartilage matrix molecules like aggrecan and collagen type II, leading to structural cartilage loss. As cartilage has very limited intrinsic repair capacity, current research focuses on techniques to prevent cartilage degeneration at an early stage or to repair degenerated cartilage tissue.

Unfortunately, the inflammatory micro-environment in OA is a major disturbing factor for cartilage homeostasis and repair. Inflammatory mediators in the OA joint dysregulate chondrocyte homeostasis and aggravate cartilage degeneration e.g. via activation of cartilage degrading enzymes [129]. In addition, OA-related inflammation can impair cartilage repair processes, such as MSC-based repair strategies currently applied in the clinic [130-133]. We previously showed that inflammatory mediators secreted by OA synovium strongly inhibit cartilage formation by human mesenchymal stem cells (MSCs) [134, 135]. Moreover, synovial fluid obtained from knee trauma patients inhibited *in vitro* chondrogenic redifferentiation of human chondrocytes [136, 137], and synovial fluid of OA and RA patients impaired chondrogenic differentiation of human subchondral progenitor cells [138]. However, the exact mechanism of how OA-related inflammation disrupts cartilage maintenance and repair is unclear.

A potential mechanism how inflammation disturbs cartilage homeostasis is via inhibition of transforming growth factor-β (TGF-β) signaling. TGF-β is an essential factor for cartilage homeostasis, and has multiple functions in cartilage [139]. In chondrocytes, TGF-β can signal via two different receptors, namely activin-like kinase (ALK)5 and ALK1, which results in activation of different intracellular signaling routes [53, 140]. Signaling via ALK5 leads to Smad2/3 recruitment and phosphorylation and mediates protective processes. Lack of intracellular TGF-β signaling via Smad3 results in chondrocyte hypertrophy and progressive, OA-like cartilage degeneration [58, 141]. In contrast, signaling via ALK1 results in phosphorylation of the Smad1/5/9 complex. The latter has been associated with deleterious processes such as the development of chondrocyte hypertrophy [37]. We previously showed that increased ALK1/ALK5 receptor ratio was associated with ageing and OA cartilage [53], suggesting that a tight balance between these signaling pathways is essential for healthy cartilage. Importantly, TGF-β stimulates production of

essential cartilage extracellular matrix (ECM) molecules like collagen type II and proteoglycans both *in vitro* and *in vivo* [61-63]. Intra-articular injection of TGF-β into murine knee joints, for example, significantly increases proteoglycan synthesis in articular cartilage [61]. Moreover, TGF-β potently stimulates chondrogenic differentiation of human MSCs, thereby promoting the formation of new cartilage [142, 143]. Together, this shows that TGF-β signaling is crucial for cartilage maintenance as well as cartilage formation.

In OA, TGF-β signaling is disturbed which contributes to the development of cartilage degeneration [37, 38]. This is possibly caused by the increased presence of inflammatory cytokines, such as IL-1β and IL-6, which can dysregulate TGF-β signaling in articular chondrocytes [75, 76, 106]. For example, IL-1β decreases TGF-β receptor II expression in articular chondrocytes resulting in inhibition of TGF-β-induced gene expression [75]. In addition, IL-1β has been reported to inhibit Smad2/3 signaling in chondrocytes via increasing expression of inhibitory Smad7 [75]. Moreover, IL-6 has been reported to inhibit TGF-β signaling in intestinal epithelial cells [108]. These inflammatory mediators activate distinct intracellular signaling routes, like the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κβ) or Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) signaling pathways [80, 144], and may affect TGF-β signaling via different mechanisms. However, one common factor is rapidly induced upon the presence of various pro-inflammatory cytokines, which is the regulatory protein suppressor of cytokine signaling 3 (SOCS3) [145, 146]. SOCS3 is a negative regulator of cytokine signaling, thereby usually preventing, but in some cases aggravating disease [147]. SOCS3 generally functions as a negative regulator of the JAK/STAT signaling pathway, by binding to the activated cytokine receptor or directly inhibiting JAK kinase activity, both resulting in restriction of STAT3 activation [147]. Moreover, SOCS3 can inhibit inflammatory IL-1β signaling by preventing TRAF-6 ubiquitination, thereby limiting activation of TAK1 [148].

Indeed, during joint inflammation, SOCS3 is highly expressed in both synovium and cartilage [116]. We previously showed that SOCS3 expression is increased in OA chondrocytes and correlates with the expression of multiple matrix-degrading enzymes [149]. However, what causes this correlation is unclear. Interestingly, there are indications that SOCS3 can alter the TGF-β signaling pathway. Deletion of SOCS3 in T-helper cells or liver tissue resulted in hyperproduction of TGF-β and TGF-β-induced fibrosis in mice [117, 118]. In addition, SOCS3 impairs TGF-β signaling in macrophages by binding to Smad3 and inhibiting its nuclear transportation and transcriptional activity [115]. Together this indicates a potential deleterious effect of SOCS3 on TGF-β function and signaling in cartilage. Therefore, we investigated whether inflammation-induced SOCS3 dysregulates TGF-β signaling in articular chondrocytes and/or TGF-β-mediated cartilage formation.

MATERIALS AND METHODS

Chondrocyte culture

Chondrocyte cell lines (H4 or G6 chondrocytes) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12,Gibco) with 5% fetal calf serum (FCS, Thermo Scientific), supplemented with 100 mg/l pyruvate (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin in standard culture conditions (5% CO_2 (v/v), 37 °C, 95% humidity). Murine H4 chondrocytes were derived from articular hip cartilage from C57BL/6 mice [150]. Human G6 chondrocytes were derived from femoral head cartilage of an anonymous OA donor, transduced with a temperature-dependent SV40 oncogene resulting in cell proliferation at 32°C, but not at 37°C [149]. G6 chondrocytes were cultured at 32°C for cell expansion, and experiments were performed at 37°C. Primary bovine chondrocytes were obtained from articular cartilage of bovine metacarpophalangeal joints (MCP). Cartilage slices were digested overnight with 1.5 mg/ml collagenase B (Roche Diagnostics) in DMEM/F12 to obtain a single cell suspension. Chondrocytes were centrifuged at 1,500 rpm for 10 min, washed with saline and resuspended in complete medium (consisting of the same components as the chondrocyte cell line medium but with 10% FCS). Bovine chondrocytes were plated at a density of 8 x 10^4 cells/cm^2 and cultured for 1 week in standard conditions [5% CO2 (v/v), 37 °C, 95% humidity] to assure cell attachment. Before start of experiments, bovine chondrocytes and chondrocyte cell lines were serum-starved overnight in FCS-free medium. For SOCS3-overexpressing studies G6- or primary bovine chondrocytes were transduced for 2 h with adenoviruses overexpressing active murine SOCS3 (AdSOCS3) or luciferase as negative control (AdLuc) with a multiplicity of infection (MOI) of 10 (G6 chondrocytes) or 200 (primary bovine chondrocytes). The SOCS3 adenoviral vector was constructed as described previously [146]. Here after, chondrocytes were cultured for a period of 48 h to ensure increased adenoviral-mediated protein expression before start of experiments. For stimulation experiments, chondrocytes were stimulated with rhTGF-β1 (Biolegend), rhIL-1β (R&D Systems), rhIL-6 (Biolegend) or OA synovium-conditioned medium (OAS-cm) for time periods and dosages indicated in figure legends. OAS-cm represents a pool of conditioned medium from seven different OA patients, produced as described previously [135]. Briefly, synovium obtained from OA patients undergoing total hip replacement was collected and cultured for 24h in DMEM with 0.1% BSA (0.3 g tissue/mL). Hereafter, supernatant was collected, debris was removed by centrifugation, and conditioned medium was stored at -20°C until further use.

Chondrogenic differentiation of MSCs

Human fetal bone-marrow derived MSCs (purchased from ScienCell Research Laboratories) were expanded for two weeks in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza) supplemented with 1% penicillin-streptomycin-glutamine (Gibco). MSCs were cultured at 37 °C with 5% CO_2 and 95% humidity until four passages, where after cells were stored in liquid N_2 until later use. For virus experiments, MSCs were transduced for 24 h with SOCS3 (LV-SOCS3)

or luciferase (LV-Luc) lentivirus with a concentration of 200 ng/1.0 x10^5 cells in MSCGM medium containing protamine sulfate (100 µg/ml; Sigma-Aldrich). The SOCS3 human transgene was inserted into lentiviral vector and lentivirus was packaged as described previously [151]. After virus transduction, medium was removed, cells were washed with saline and expanded for two days. To start chondrogenic differentiation, MSCs were centrifuged at 300 *g* for 8 min in polystyrene V-bottom tubes (Greiner Bio-One) to obtain high-cell density pellets (200.000 cells/pellet). MSC-pellets were cultured for one week in 500 µl chondrogenic medium, containing Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 1% penicillin-streptomycin-glutamine (Gibco), insulin (6.25 µg/mL), transferrin (6.25 µg/mL), sodium selenite (6.25 ng/mL), proline (0.4 mg/mL), sodium pyruvate (1 mg/mL), linoleic acid (5.35 µg/mL), ascorbic acid (50 µg/mL), dexamethasone (10^{-7} M), and bovine serum albumin (1 mg/mL; all from Sigma-Aldrich). In addition, rhTGF-β1 (10 ng/ml) and rhBMP-2 (20 ng/ml) were added to chondrogenic medium, and freshly added during refreshment of complete chondrogenic medium (three times per week).

Histological staining and measurement of GAG content in MSC pellets

For measurements of glycosaminoglycan (GAG) content, individual pellets were digested overnight using 0.1% (w/v) papain (Sigma-Aldrich) in digestion buffer (200 mM $NaPO_4$, 100 mM NaAc, 5 mM cysteine-HCl, 10 mM EDTA at pH 6.4) at 60 °C. Hereafter, sulfated GAG content was measured in 40 µl of papain-digested sample by addition of 200 µl of dimethylmethylene blue (DMB) solution (0.05 mM DMB, 41 mM NaCl, 45 mM glycine, at pH 3.0) following the Farndale assay [152]. Absorbance was measured at 595 nm using the iMark-Reader (Bio-Rad). For histological staining, pellets were fixed in 4% formalin overnight, dehydrated and embedded in paraffin. Hereafter, tissue sections of 5 µm were prepared. To visualize sulfated GAGs, sections were stained with aqueous Safranin-O and counterstained with aqueous Fast Green (0.1%, Brunschwig Chemie). Pellet size was evaluated by eye on a macroscopical level.

Protein isolation and western blot

Chondrocytes were lysed using lysis buffer (Cell Signaling) containing protease inhibitor cocktail (PIC, complete, Roche Diagnostics). Cell lysates were sonicated on ice for 10 cycles of 30 seconds sonication using a Bioruptor (Diagenode) as described previously [153]. Protein concentration was normalized between samples using a bicinchoninic acid assay (BCA, Thermo Scientific). Proteins were separated with a 10% reducing bis-acrylamide SDS-PAGE gel and transferred to a 0.45 µm pore nitrocellulose membrane (Life Sciences) via wet transfer (Towbin buffer, 2 h, 275 mA at 4 °C). Membranes were incubated overnight at 4 °C with primary antibodies directed against SOCS3 (polyclonal rabbit antibody, #2923S, 1:1000, Cell Signaling), p-STAT3 (polyclonal rabbit antibody, #9131, 1:1000, Cell Signaling), p-Smad3 (monoclonal rabbit antibody, #C25A9, 1:000, Cell Signaling), p-Smad1/5/9 (polyclonal rabbit antibody, #9511, 1:000, Cell Signaling), MBD3 (monoclonal mouse antibody, 10281, 1:1000, IBL), β-actin (monoclonal rabbit

antibody, #4970, 13E5, 1:1000, Cell Signaling) or GAPDH (mouse monoclonal antibody, 1G5, 1:10,000 Sigma Aldrich). After overnight incubation, membranes were incubated for 1 h with polyclonal Goat anti-Rabbit coupled to horseradish peroxidase (1:1500, Dako) at RT. Enhanced chemiluminescence (ECL) was used to visualize proteins with ECL prime kit (GE Healthcare) and ImageQuant LAS4000 (Leica) according to manufacturer's instructions.

Enrichment of nuclear and cytoplasmic fractions

To investigate p-Smad3 nuclear translocation, nuclear and cytoplasmic protein fractions were enriched as described previously [154]. Harvested chondrocytes were incubated for 10 min on ice in buffer A: 10 Mm HEPES (Sigma-Aldrich), 10 mM KCl (Merck), 0.5 mM DTT (Sigma-Aldrich), 1.5 mM $MgCl_2$ (Merck), 0.05% NP40 (BDH), pH 7.9 supplemented with 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1 mM Na_3VO_4 (all from Sigma-Aldrich) and PIC (Roche Diagnostics). Protein lysates were centrifuged at 400 *g* for 10 min at 4 °C in a tabletop centrifuge (Eppendorf). Supernatant was collected (cytoplasmic fraction) and stored. Remaining pellet (nuclear fraction) was washed in buffer A, spun down and resuspended in buffer B (5 mM HEPES, 0.5 mM DTT, 0.2 mM EDTA (Merck), 300 mM NaCl, 1.5 mM $MgCl_2$, 26% glycerol (v/v, Merck), pH 7.9) supplemented with phosphatase and protease inhibitors. Nuclear fractions were transferred to a Dounce homogenizer and nuclei were lysed using a glass type B (tight) pestle (Sigma-Aldrich). Nuclear fraction was incubated for 1 h at 4 °C on a roller bench, centrifuged at 3,200 *g* for 20 min at 4 °C, where after supernatant (nuclear fraction) was collected and stored. Before western blotting, nuclear and cytoplasmic fractions were sonicated according to normal protocol (described in western blot section) and protein concentration was determined with BCA measurement. MBD3 and β-actin were used as respective (loading) controls for nuclear and cytoplasmic fraction enrichment.

TGF-β transcriptional reporter luciferase assays

TGF-β transcriptional activity was determined using luciferase reporter assays. The effect of OA-related inflammation on TGF-β transcriptional activity was investigated using the pNL1.2-SBE luciferase reporter assay in G6 chondrocytes. This reporter assay consists of a SMAD binding element (SBE) driven by a minimal promoter including a TATA-box (bold) (Genecust) [155]. The SBE-DNA fragments contains three palindromic SMAD3 binding elements (underlined): 5'AGTAT<u>GTCTAGAC</u>TGAAGTAT<u>GTCTAGAC</u>TGAAGTAT<u>GTCTAGAC</u>TGACTCGAGGATATCAA GATCTGGCCTCGGCGGCCTAGATGAGACACT**AGAGGGTATATAATGGAAGCTCGACTT CCAG**-3'. Promoter sequences were cloned into the pNL1.2 reporter vector as follows. First, the SBE-fragment was digested with KpnI/HindIII restriction enzymes (NEB)-and ligated with T4 Ligase (NEB) into the KpnI/HindIII-digested pNL1.2 vector (Promega). Hereafter, correct orientation was confirmed with restriction analysis. To obtain plasmid DNA, TOP10-transformed competent cells were propagated and plasmid DNA was extracted using a Plasmid Maxi Kit (Qiagen) according to manufacturer's protocol. Construct sequences were validated by Sanger sequencing. G6

chondrocytes were seeded in a cell density of 8 x 10^4 cells/cm^2 and transfected with the pNL1.2-SBE luciferase reporter plasmid using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's protocol. The effect of SOCS3 on TGF-β transcriptional activity was determined by co-transduction of G6 chondrocytes with the CAGA12-luciferase reporter adenovirus (26), combined with the SOCS3 adenovirus (both MOI 5). To determine luciferase activity of reporter assays, G6 chondrocytes were lysed using reporter assay lysis buffer (Promega) and luciferase activity was measured after addition of Bright Glo TM substrate (CAGA-Luc) or Nano Glo (pNL1.2-SBE) (both from Promega) using a luminometer (Clariostar, BMG).

RNA isolation and quantitative polymerase chain reaction (PCR) analysis

Total messenger RNA was extracted from chondrocytes using TRIzol reagent (Sigma Aldrich) following manufacturer's protocol. After isolation, RNA was dissolved in ultra-pure water and concentration and purity was measured using a NanoDrop photospectrometer (Thermo Scientific). Hereafter, a maximum of 1 µg of mRNA was treated with 1 Unit DNAse (Life Technologies) for 15 min at RT to remove possible genomic DNA, followed by an inactivation step with 1 µL 25 mM EDTA (Life Technologies) at 65°C for 10 min. An equal amount of RNA was reverse transcribed to complementary DNA using 1.9 µL ultrapure water, 2.4 µl 10x DNAse buffer, 2.0 µl 0.1M dithiothreitol, 0.8 µl 25mM dNTP, 0.4 µg oligo dT primer, 200U M-MLV reverse transcriptase (Life Technologies) and 0.5 µl 40 U/ml RNAsin (Promega). Reverse transcription protocol was as follows: 5 min at 25°C, 60 min at 39°C, and 5 min at 95°C using a thermos-cycler. Gene expression was measured using SYBR Green Master Mix (Applied Biosystems) and 0.25 mM primers (Biolego, see Table 1) using a StepOnePlus real-time PCR system (Applied Biosystems). Amplification protocol: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting curves were analyzed to confirm product specificity. To calculate the relative gene expression (−ΔCt), the average of two reference genes was used: *hGAPDH* and *hRPS27A* for G6 chondrocytes and *bGAPDH* and *bRPS14* for bovine chondrocytes.

Statistical analysis

Quantitative data were expressed as curve or column scatter with displayed mean ± standard deviation (SD). Mean represents either mean of multiple experiments, or mean of technical replicates as indicated in Figure legends. Before each analysis, Gaussian distribution was tested using the D'Agostino-Pearson Omnibus K2 test. Differences in sGAG content of MSC-pellets were tested using one-way analysis of variance (ANOVA) with Bonferroni post-correction to correct for multiple comparisons. The effect of inflammatory mediators or SOCS3 overexpression on TGF-β transcriptional activity was determined with Kruskal Wallis analysis, followed by the Dunn's Multiple Comparison Test. Gene expression was analyzed with Kruskal Wallis and Dunn's post-test (differences in ALK expression) or with One-Way ANOVA followed by Bonferroni's post-test (differences in TGF-β-mediated induction of immediate early genes) or Dunnett's post-test (effect of inflammation on *hSOCS3* expression). Statistical analyses were performed using

GraphPad Prism version 5.03 (GraphPad Software) on the absolute measured values. P-values <0.05 were considered significant.

Table 1. Template and sequence of the primers used in this study.

Gene	Forward Sequence (5' -> 3')	Reverse Sequence (5' -> 3')
hGAPDH	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTGAGC
hRPS27a	GTTAAGCTGGCTGTCCTGAAA	CATCAGAAGGGCACTCTCG
hALK5	CGACGGCGTTACAGTGTTTCT	CCCATCTGTCACACAAGTAAAATTG
hALK1	GACTCAAGAGCCGCAATGTG	GGTCGGCGATGCAACAC
hSERPINE1	GTCTGCTGTGCACCATCCCCCATC	TTGTCATCAATCTTGAATCCCATA
hSMAD7	CCTTAGCCGACTCTGCGAACTA	CCAGATAATTCGTTCCCCCTGT
hJUNB	AACAGCCCTTCTACCACGAC	CAGGCTCGGTTTCAGGAGTT
hID1	ACGATCGCATCTTGTGTC	CTTGTTCTCCCTCAGATCC
h/bSOCS3	TCGGACCAGCGCCACTT	CACTGGATGCGCAGGTTCT
bGAPDH	CACCCACGGCAAGTTCAAC	TCTCGCTCCTGGAAGATGGT
bRPS14	CATCACTGCCCTCCACATCA	TTCCAATCCGCCCAATCTTCA
bALK5	CAGGACCACTGCAATAAAATAGAACTT	TGCCAGTTCAACAGGACCAA
bALK1	ACAACACAGTGCTGCTCAGACA	TGCTCGTGGTAGTGCGTGAT
bSERPINE1	CGAGCCAGGCGGACTTC	TGCGACACGTACAGAAACTCTTGA
bSMAD7	GGGCTTTCAGATTCCCAACTT	CTCCCAGTATGCCACCACG
bJUNB	CCTTCTACCACGACGACTCA	CCGGGTGCTTTGAGATTTCG
bID1	GCTCCGCTCAGCACTCTCAA	GATCGTCCGCTGGAACACA

RESULTS

OA-related inflammation induces SOCS3 expression and inhibits TGF-β transcriptional activity in articular chondrocytes

The cytokine-inducible protein SOCS3 has been identified as a potential inhibitor of TGF-β/Smad3 signaling in macrophages [115], but whether SOCS3 impairs protective TGF-β signaling in articular chondrocytes is yet unknown. As SOCS3 is a regulator of inflammation in cells of the immune system [147], we first established whether inflammatory factors modulate SOCS3 gene and protein expression in articular chondrocytes. Stimulation with rhIL-1β, rhIL-6 or OAS-cm for 6 h strikingly increased gene expression of *hSOCS3* in G6 chondrocytes with 6.5-fold (2.7 ΔCt), 2.0-fold (1.0 ΔCt), and 6.1-fold (2.6 ΔCt) respectively (Fig. 1A). In contrast with the effect of inflammatory mediators, we found no evidence that *hSOCS3* gene expression was affected by stimulation with rhTGF-β. Western blot confirmed that IL-1β also increased SOCS3 protein expression in articular chondrocytes, while TGF-β had no effect (Fig. 1B). A combination of these factors did not lead to an additional effect on SOCS3 expression (data not shown). Now that we

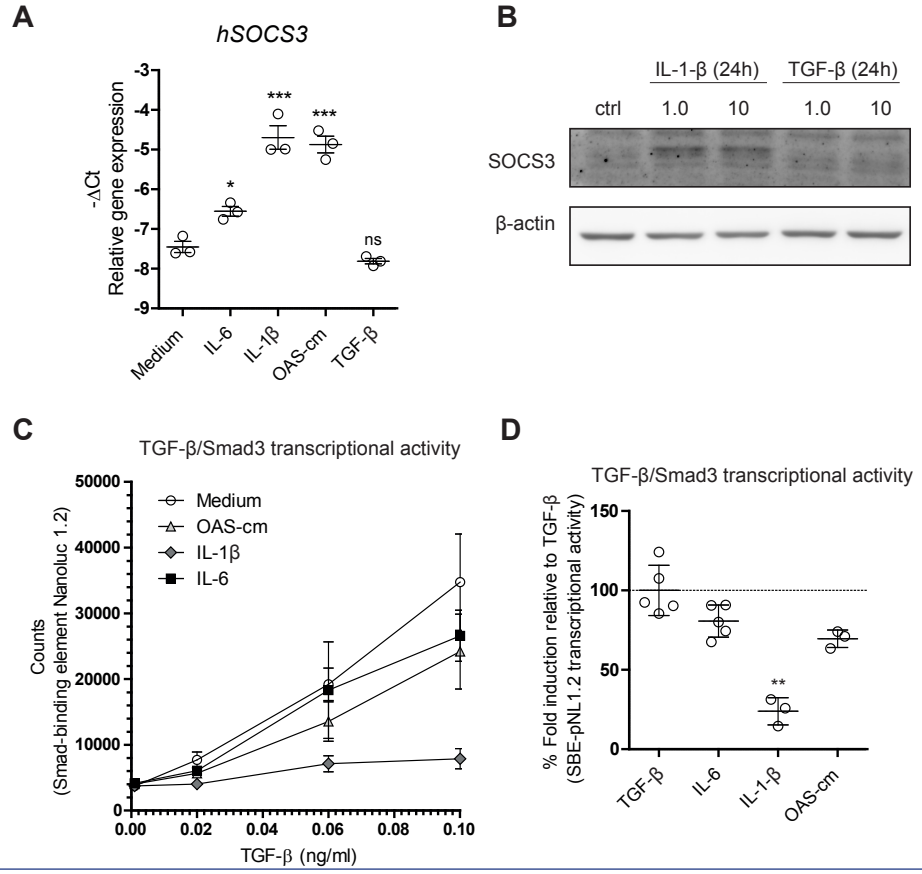


Figure 1. OA-related inflammatory factors increase SOCS3 expression and simultaneously inhibit TGF-β transcriptional activity in articular chondrocytes. (A) G6 chondrocytes were stimulated in duplicate or triplicate with rhIL-6 (25 ng/ml), rhIL-1β (1.0 ng/ml), OAS-cm (2.5%) or rhTGF-β (1.0 ng/ml) for 6 h, where after hSOCS3 gene expression was analyzed using qPCR. Data are expressed as mean ± SD of technical replicates. **(B)** H4 chondrocytes were stimulated with rhIL-1β (1.0, 10.0 ng/ml) or rhTGF-β (1.0, 10.0 ng/ml) to study the effect on SOCS3 protein expression as determined by western blot. **(C)** To study interaction between OA-related inflammation and functional TGF-β signaling, G6 chondrocytes were transfected with a TGF-β transcriptional reporter assay construct (SBE-pNL1.2 luciferase reporter). After transfection, G6 chondrocytes were pre-incubated overnight with rhIL-6 (25 ng/ml), rhIL-1β (1.0 ng/ml) or OAS-cm (0.5%) and thereafter stimulated with increasing concentrations of rhTGF-β for 5 h. Data represents mean ± SD of three technical replicates. **(D)** Luciferase activity relative to experimental condition stimulated with rhTGF-β (0.1 ng/ml), as set at 100%. Data represents mean ± SD of two independent experiments performed in duplicate or triplicate. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ as measured by one-way ANOVA **(A)** or Kruskall Wallis test **(D)** both followed by Dunn's multiple comparison test.

established that OA-related inflammation increases SOCS3 expression in articular chondrocytes, we investigated if these inflammatory mediators inhibit TGF-β signaling at the same time. To study TGF-β signaling, we used a luciferase reporter assay with SMAD-binding elements. Luciferase activity was strongly induced in a dose-dependent fashion in G6 chondrocytes, with a maximum of ~8.5-fold induction at 0.1 ng/ml of rhTGF-β (Fig. 1C). Interestingly, TGF-β-induced luciferase activity was counteracted by pre-incubation with the pro-inflammatory mediators IL-1β, IL-6 and OAS-cm (Fig. 1C). More specifically, transcriptional activity induced by 0.1 ng/ml of TGF-β was significantly inhibited by IL-1β with approximately 80%, while a downward trend was observed in response to OAS-cm (~30%) and IL-6 (~20%)(Fig. 1D). Since these inflammatory mediators highly increase SOCS3 expression at the same time, and SOCS3 was previously shown to inhibit TGF-β/Smad3 signaling in other cell types, it seemed plausible that inflammation-induced SOCS3 has a central role in reducing TGF-β signaling in this setting.

Overexpression of SOCS3 does not alter TGF-β transcriptional activity or C-terminal phosphorylation of Smad3

To determine whether SOCS3 could be pivotal in the inhibitory effect of inflammatory mediators on TGF-β function, increased SOCS3 expression was mimicked using adenoviral overexpression. In order to study TGF-β transcriptional activity, G6 chondrocytes were simultaneously transduced with the CAGA12-luciferase adenovirus in combination with a SOCS3- or control adenovirus. As expected, stimulation with rhTGF-β strongly induced luciferase activity with a maximum of ~18-fold (Fig. 2A). However, overexpression of SOCS3 did not significantly affect TGF-β-induced luciferase production compared to control conditions. This lack of effect was not due to unsuccessful overexpression, since SOCS3 protein levels were highly increased in G6 chondrocytes after transduction visualized with western blot (Fig. 2B). Moreover, endogenous p-STAT3 was clearly suppressed by SOCS3 overexpression, confirming transgene activity (Fig. 2B). Previously, it was shown that SOCS3 can directly inhibit Smad3 phosphorylation [115]. Therefore, we investigated if SOCS3 might inhibit TGF-β signaling on a more upstream level by studying C-terminal phosphorylation of Smad3 at several time points after TGF-β stimulation using western blot. However, SOCS3 overexpression did not distinctly alter p-Smad3 levels at baseline or after TGF-β stimulation (Fig. 2C). Of note, we observed a small reduction in p-Smad3 levels at 2 h after TGF-β stimulation in SOCS3 overexpression conditions compared to control virus. However, this effect was not consistent and not detectable at other time points. As it was previously shown that SOCS3 can inhibit nuclear translocation of Smad3 in macrophages [115], we additionally studied p-Smad3 levels in nuclear and cytoplasmic protein fractions of SOCS3-transduced G6 chondrocytes. Levels of p-Smad3 were detected in both cytoplasmic and nuclear fractions, and were clearly induced in nuclear fractions after 1 h stimulation with rhTGF-β (Fig. 2D). However, we observed no effect of SOCS3 overexpression on p-Smad3 nuclear translocation in control or TGF-β stimulated conditions. Together, these results indicate that increasing SOCS3 does not affect TGF-β/Smad3 transcriptional activity or Smad3 C-terminal phosphorylation in articular chondrocytes.

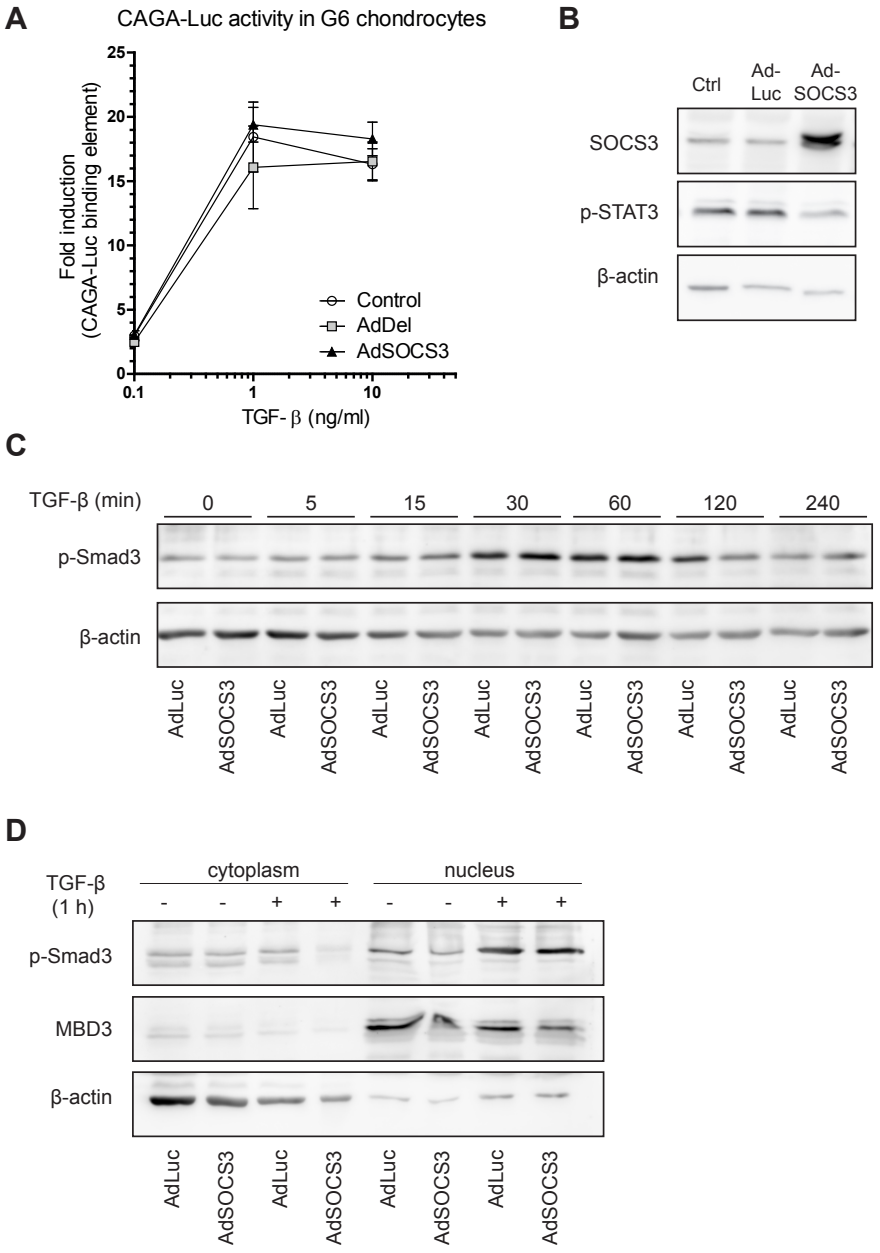


Figure 2. Overexpression of SOCS3 does not affect TGF-β transcriptional activity, C-terminal phosphorylation or nuclear activation of Smad3. To study the effect of SOCS3 on TGF-β signaling, G6 chondrocytes were transduced with SOCS3- or control adenovirus (luciferase (Luc) or empty backbone (Del)), or left untreated (control). At 48 h after transduction, G6 chondrocytes were stimulated with rhTGF-β

and several read-out parameters were analyzed. **(A)** Transcriptional activity in SOCS3- versus control conditions was determined by adenoviral transduction with CAGA12-luc construct. G6 chondrocytes were stimulated with increasing concentrations of rhTGF-β for 16 h and luciferase activity was determined. Data is expressed relative to medium control and presented as mean ± SD of four technical replicates and is representative of three independent experiments. **(B)** SOCS3 protein expression 48h after adenoviral transduction with SOCS3- or luciferase adenovirus as determined by western blot. Additionally, p-STAT3 levels were analyzed to verify transgene activity. **(C)** C-terminal phosphorylation of Smad3 was analyzed in SOCS3 overexpression- and control conditions at several time points (0, 5, 15, 30, 60, 120, 240 min) after stimulation with 1.0 ng/ml of rhTGF-β using western blot. **(D)** Nuclear translocation of p-Smad3 was analyzed after 1h of rhTGF-β (1 ng/ml) stimulation by enrichment of nuclear and cytoplasmic protein fractions. β-actin was used as loading control for cytoplasmic fraction and MBD3 for the nuclear fraction. Statistical differences in CAGA-12 luc assay were tested using the Kruskall Wallis test followed by Dunn's multiple comparison test.

SOCS3 does not change expression levels of TGF-β receptors ALK1 and ALK5 or activation of Smad2/3 and Smad1/5/9 signaling pathways

TGF-β can activate two different type I receptors in chondrocytes, named ALK5 and ALK1, which both activate a different intracellular Smad signaling route [53]. Therefore, a possible alternative mechanism how SOCS3 may alter TGF-β signaling could be via modulation of the balance between ALK1 and ALK5 receptors. However, gene expression of *hALK5* and *hALK1* was not affected by overexpression of SOCS3 in G6 chondrocytes (Fig. 3A). Also in primary bovine chondrocytes, SOCS3 overexpression did not affect bALK5 or bALK1 expression, confirming that this absence of effect was not due to the choice of cell source (Supplementary Fig. 1A). As SOCS3 may influence Smad signaling downstream of the receptors, we also determined TGF-β-mediated activation of p-Smad2/3 versus p-Smad1/5/9 signaling pathway in G6- and bovine chondrocytes. Increased phosphorylation of both pathways was clearly visible after 1 h of TGF-β stimulation in articular chondrocytes. Nonetheless, TGF-β-induced phosphorylation of Smad2/3 and Smad1/5/9 was not affected by SOCS3 overexpression in both G6- and primary bovine chondrocytes (Fig. 3B, Supplementary Fig. 1B). These results indicate that the balance in ALK5-Smad2/3 and ALK1-Smad1/5/9 signaling is not affected by SOCS3 in articular chondrocytes.

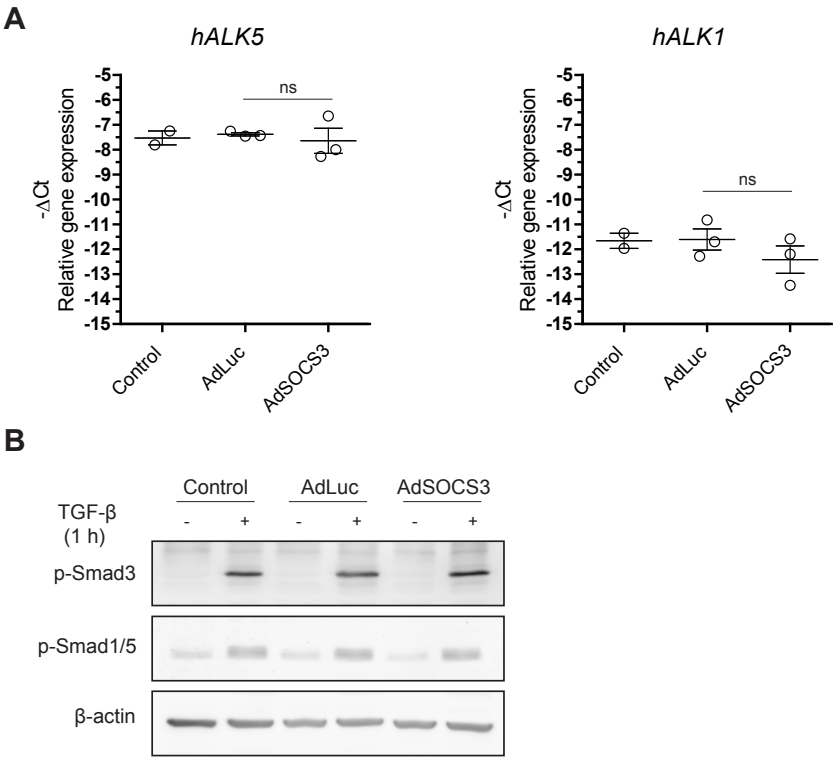


Figure 3. ALK1 and ALK5 expression and signaling is not affected by SOCS3 overexpression. To study the effect of SOCS3 on TGF-β receptor expression and signaling, G6 chondrocytes were transduced with SOCS3- or luciferase adenovirus, or left untreated (control). **(A)** Gene expression of hALK1 and hALK5 receptors was analyzed 48 h after virus transduction using qPCR. Data represents mean of three independent experiments, in which each point represents the mean of three technical replicates. **(B)** C-terminal phosphorylation of Smad3 or Smad1/5/9 was analyzed with western blot in G6 chondrocytes at 1 h after rhTGF-β stimulation (1.0 ng/ml). Statistical differences in hALK1 and hALK5 expression were tested with Kruskall Wallis followed by Dunn's multiple comparison test.

TGF-β-dependent target gene expression is not altered by enhanced SOCS3 in articular chondrocytes

To study the effect of SOCS3 on TGF-β-mediated transcription in a more physiological setting, we analyzed expression patterns of Smad3-dependent target genes *hSERPINE1, hJUNB*, *hSMAD7* and *hID-1* in G6 chondrocytes [156-160]. Stimulation with 1 ng/ml of rhTGF-β markedly increased gene expression of *hSERPINE1* (4.6-fold, 2.2 ΔCt) *, hJUNB* (5.6-fold, 2.5 ΔCt), *hSMAD7* (5.6-fold, 2.5 ΔCt) and *hID-1* (2.5-fold,1.3 ΔCt) within 2 h after stimulation (Fig. 4). Adenoviral overexpression of SOCS3 strongly increased *hSOCS3* gene expression with ~2000-fold (11.3 ΔCt) compared to the luciferase control (Supplementary Fig. 2A). However, SOCS3

overexpression did not significantly change TGF-β-mediated induction of *hSERPINE1, hJUNB*, *hSMAD7* or *hID-1* in G6 chondrocytes (Fig. 4). Also in primary bovine chondrocytes, increased SOCS3 did not modify TGF-β-dependent expression of these target genes as measured at 2 h, 8 h and 24 h after stimulation (Supplementary Fig. 2B). Together, this indicates that increased SOCS3 expression does not impair TGF-β-induced expression of Smad3-dependent genes in articular chondrocytes.

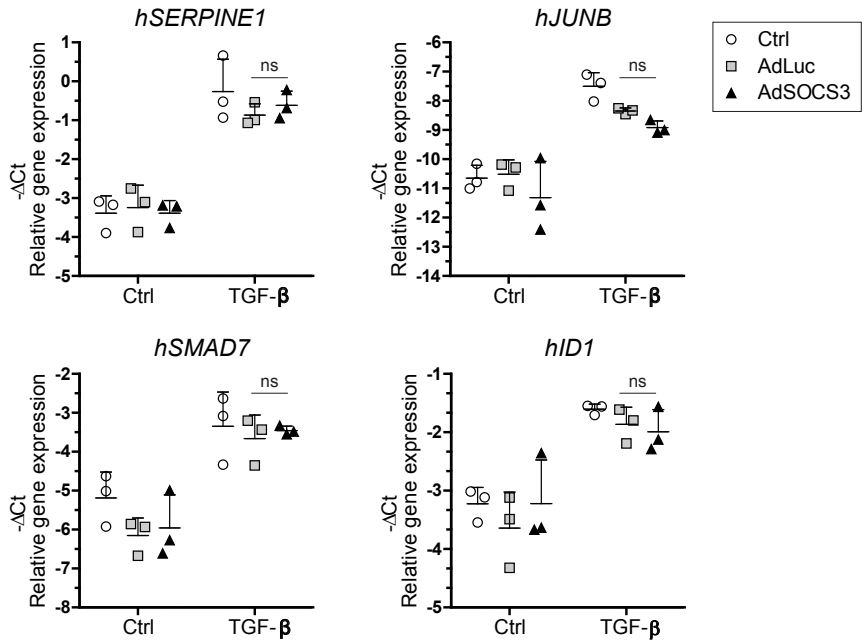


Figure 4. Adenoviral overexpression of SOCS3 does not alter TGF-β-mediated induction of Smad3-dependent genes. To study the effect of SOCS3 on TGF-β-induced expression of Smad3-dependent genes, G6 chondrocytes were transduced with SOCS3- or luciferase adenovirus, or left untreated (no virus control). After 48 h, G6 chondrocytes were stimulated with rhTGF-β for 2 h and expression of hSERPINE1, hJUNB, hSMAD7, and hID1 was analyzed with qPCR. Data represents mean of three independent experiments, in which each point represents the mean of three technical replicates. Differences in TGF-β-mediated induction of hSERPINE1, hJUNB, hSMAD7 and hID1 were tested with one-way ANOVA and Bonferroni post-hoc test.

Enhanced SOCS3 expression impairs TGF-β-dependent MSC-based cartilage formation

We have previously shown that an inflammatory OA-like microenvironment strongly reduces TGF-β-dependent cartilage formation of MSC pellets [134, 135]. To study whether inflammation-induced SOCS3 might be involved in this inhibitory effect, we first determined whether OA-related inflammation increases SOCS3 expression in MSCs. Indeed, protein expression of SOCS3 was induced by OAS-cm treatment in chondrogenic MSC-pellets, especially after 96h incubation (Fig. 5A). To study the role of SOCS3 in TGF-β-dependent cartilage formation, MSCs were transduced with either SOCS3- or luciferase lentivirus, and cartilage formation was stimulated by 3D-pellet formation and addition of chondrogenic medium supplemented with rhTGF-β (10 ng/ml) and rhBMP-2 (20 ng/ml) (Fig. 5B). Overexpression of SOCS3 on western blot was confirmed after 1 week of culture (Fig 5C). Moreover, SOCS3 overexpression resulted in inhibition of endogenous p-STAT3 levels, which demonstrates adequate transgene activity (Fig. 5C). Interestingly, MSC-pellet size was strongly reduced by SOCS3 overexpression after 7 days of chondrogenic differentiation (Fig. 5D). Moreover, intensity of Safranin-O staining was reduced in SOCS3-overexpressing pellets, indicating decreased sulphated GAGs content. Indeed, exact measurement of sGAG-content showed a significant reduction of ~50% in sGAG content of SOCS3-pellets compared to luciferase control (Fig. 5E). Similar to the results obtained in chondrocytes, SOCS3 overexpression did not affect p-Smad3 levels in MSC-pellets compared to luciferase control in the presence of TGF-β (Fig. 5F). Altogether, this shows that increased SOCS3 expression inhibits MSC-based cartilage formation.

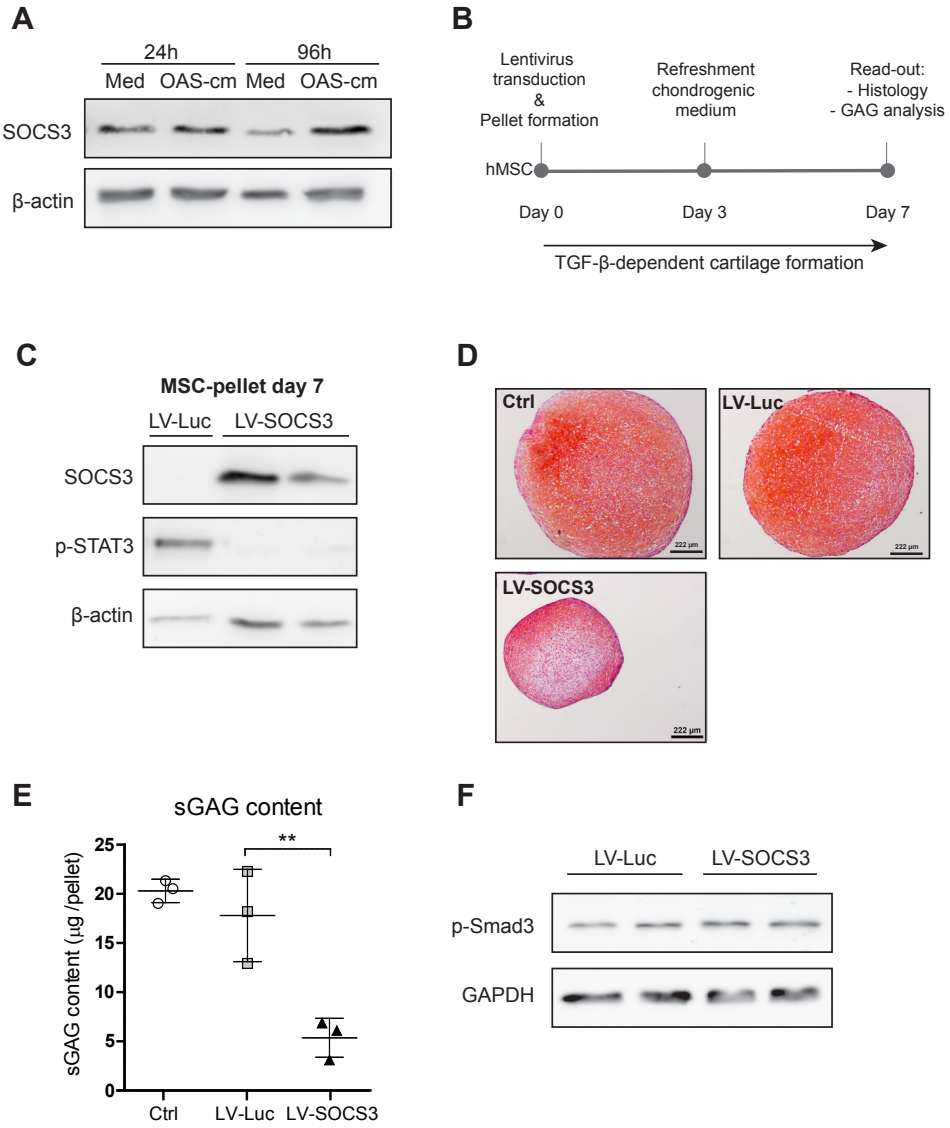


Figure 5. Lentiviral overexpression of SOCS3 inhibits MSC-based cartilage formation. (A) Endogenous protein expression of SOCS3 protein in MSC-pellets after 7 days of chondrogenic differentiation as determined by western blot. Pellets were stimulated with medium only or OAS-cm (2.5%) for a period of 24 h or 96 h. **(B)** Overview of experimental design. MSCs were transduced with SOCS3 or luciferase lentivirus and hereafter cultured in chondrogenic medium containing rhTGF-β (10 ng/ml). After 7 days pellets were harvested for histological analysis and sGAG measurement. **(C)** Protein levels of SOCS3 and p-STAT3 as determined by western blot in MSCs at 1 week after lentiviral transduction with SOCS3- or luciferase lentivirus. **(D)** Histological staining of MSC-pellets at day 7 of chondrogenic differentiation, transduced with SOCS3- or luciferase lentivirus. Intensity of safranin-O staining indicates proteoglycan content of MSC-

pellets. Representative pictures are shown of three pellets per condition. **(E)** Quantification of sGAG content of MSC pellets at day 7 after differentiation in MSCs with no virus (Ctrl), or transduced with SOCS3- or luciferase lentivirus. Data represents mean of three independent experiments, in which each point is the mean of three pellets. **(F)** C-terminal phosphorylation of Smad3 determined by western blot in MSCs at 1 week after lentiviral transduction with SOCS3- or luciferase lentivirus. ** = $P < 0.01$ as measured by one-way ANOVA with Bonferonni post-hoc test.

DISCUSSION

The inflammatory micro-environment in the OA joint impairs TGF-β signaling in chondrocytes as well as TGF-β-dependent cartilage repair processes, which may be regulated via the central cytokine-inducible protein SOCS3. In this study, we show that OA-related inflammatory factors indeed increase SOCS3 expression in articular chondrocytes, and simultaneously inhibit TGF-β transcriptional activity. However, inflammation-mediated inhibition of TGF-β signaling in chondrocytes is not mediated via SOCS3, as we found no evidence that SOCS3 alters TGF-β receptor expression, Smad phosphorylation and nuclear translocation, or TGF-β-mediated transcription. Finally, we demonstrate that SOCS3 overexpression strongly inhibits MSC-based cartilage formation, resulting in decreased pellet size and sGAG content. Based on our findings in chondrocytes, it is likely that this is independent of SOCS3 directly affecting TGF-β signaling.

To study the effect of OA-related inflammation on *hSOCS3* expression in articular chondrocytes, we stimulated chondrocytes with IL-6, IL-1β or OAS-cm. IL-6 is a common inducer of SOCS3, and IL-6 levels are highly increased in OA synovial fluid [161-163]. IL-1β is a pro-inflammatory cytokine present in OA synovial fluid, which can directly inhibit proteoglycan synthesis, increase expression of matrix degenerative mediators, and regulate SOCS3 expression [134, 146, 149, 164, 165]. Additionally, conditioned medium derived from OA-synovial biopsies (OAS-cm) was used, which reflects the complex inflammatory microenvironment present in an OA joint. Our results showed that IL-6, IL-1β and OAS-cm all induced *hSOCS3* expression after 5 h, and these effects were most prominent after IL-1β or OAS-cm stimulation. This is in line with our previous findings that IL-1β potently increases SOCS3 gene and protein expression in both H4 and G6 chondrocytes, although IL-6 and OAS-cm were not included in these studies [146, 149]. Possibly, IL-6 causes higher induction of SOCS3 at earlier time points, as computational modeling shows that IL-6 stimulation can result in rapid and transient activation of SOCS3 [166]. Moreover, our results showed that the inflammatory mediators IL-1β, IL-6 and OAS-cm, besides increasing SOCS3 expression, also inhibit TGF-β transcriptional activity in articular chondrocytes. Together, this initially suggested that inflammation-induced SOCS3 might have been a central mediator in inflammation-mediated inhibition of TGF-β signaling in cartilage. However, our data did not show that SOCS3 inhibits TGF-β signaling in articular chondrocytes.

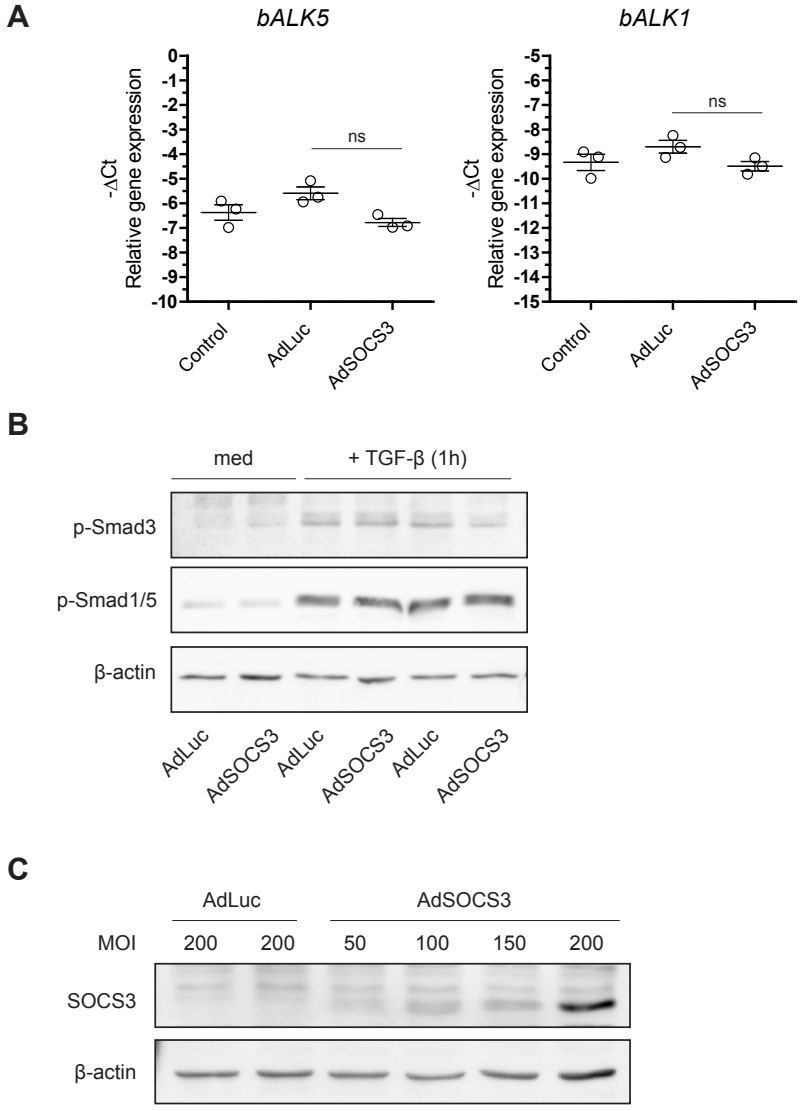
In articular cartilage, multiple functions of SOCS3 have been reported, both protective as well as deleterious. For instance, a key role for SOCS3 in controlling chondrocyte cytokine responses during joint inflammation has been demonstrated [84]. In cartilage explants where SOCS3 was deleted (SOCS3$^{-/-}$), stimulation with the inflammatory cytokine IL-6 resulted in increased expression of catabolic mediators and enhanced cartilage degradation. Moreover, intra-articular injection of inflammatory mediators such as IL-6 in cartilage specific SOCS3$^{-/-}$ mice aggravated cartilage damage and inflammation [84]. This suggests that SOCS3 has a beneficial role in chondrocytes, by restricting pro-inflammatory cytokine signaling and thereby the development of cartilage damage. On the other hand, deleterious functions of SOCS3 in chondrocytes have also been reported. We previously showed that SOCS3 inhibits insulin growth factor-1 (IGF-1) signaling in chondrocytes, via the reduction of insulin receptor substrate-1 phosphorylation [146]. As IGF-1 is an essential stimulator of proteoglycan synthesis in cartilage [167, 168], SOCS3 may cause non-responsiveness to IGF-1 via this mechanism, leading to a reduced anabolic capacity in chondrocytes. In addition, SOCS3 can inhibit TGF-β signaling in macrophages via direct inhibition of Smad3 phosphorylation and nuclear translocation [115]. Therefore, we initially anticipated this could reflect a new detrimental role for SOCS3 in cartilage, via the inhibition of protective TGF-β signaling in chondrocytes. However, increasing SOCS3 levels using viral overexpression did not affect TGF-β-induced transcriptional activity, C-terminal phosphorylation- or nuclear translocation of Smad3 in human or bovine articular chondrocytes. This finding is opposed to the observations of Liu et al., who showed that TGF-β directly binds Smad3 and inhibits Smad3 activation and nuclear translocation to control Toll like receptor 4 (TLR4) signaling in macrophages [115]. In contrast to our approach, they studied the role of endogenously expressed SOCS3 using small interfering (si)RNA-based silencing of SOCS3. However, in our experience siRNA-transfection is difficult to employ in chondrocytes as they secrete matrix macromolecules during culture resulting in low transfection efficiency [169, 170], which is why we used a viral overexpression strategy instead. Moreover, we studied the effect of SOCS3 on several aspects of the TGF-β signaling pathway, and found no effect on expression of TGF-β receptors ALK5/ALK1, respective activation of p-Smad2/3 and p-Smad1/5/9 signaling or TGF-β-dependent expression of Smad3-dependent genes. Together with the lack of effect on Smad3 phosphorylation and nuclear translocation, these results show that SOCS3 does not impair TGF-β signaling in articular chondrocytes. Possibly, the effect of SOCS3 on TGF-β/Smad3 signaling is cell type-dependent, and may be present in other cell types such as macrophages [115].

Interestingly, overexpression of SOCS3 did strongly inhibit chondrogenic differentiation of MSCs. As the presence of inflammatory mediators is a known limiting factor of MSC-based cartilage repair, it is possible that inflammation-induced SOCS3 hampers cartilage repair strategies in OA patients. Although TGF-β is a crucial factor for our chondrogenic differentiation model [171], we convincingly showed that SOCS3 did not inhibit TGF-β signaling in chondrocytes.

We therefore suggest that the inhibitory effect of SOCS3 on MSC-based cartilage formation is mediated via direct inhibition of the IGF-1 pathway. IGF-1 is crucial for the synthesis of essential matrix components during chondrogenesis [167, 168], and it was previously shown that SOCS3 inhibits IGF-1 signaling in chondrocytes [146]. Alternatively, SOCS-3 may impair cartilage formation via inhibition of its natural target STAT3. Activation of STAT3 signaling strongly stimulates chondrogenic differentiation and increases matrix production [172]. Moreover, IL-6/STAT3 signaling has been shown to stimulate cartilage regeneration [96]. Inhibition of SOCS3 in MSCs may ultimately improve cartilage repair in an inflammatory OA joint. But first, we need to make sure that targeting of SOCS3 during cartilage formation is only beneficial, and does not result in uncontrolled cytokine signaling in inflammatory conditions [147].

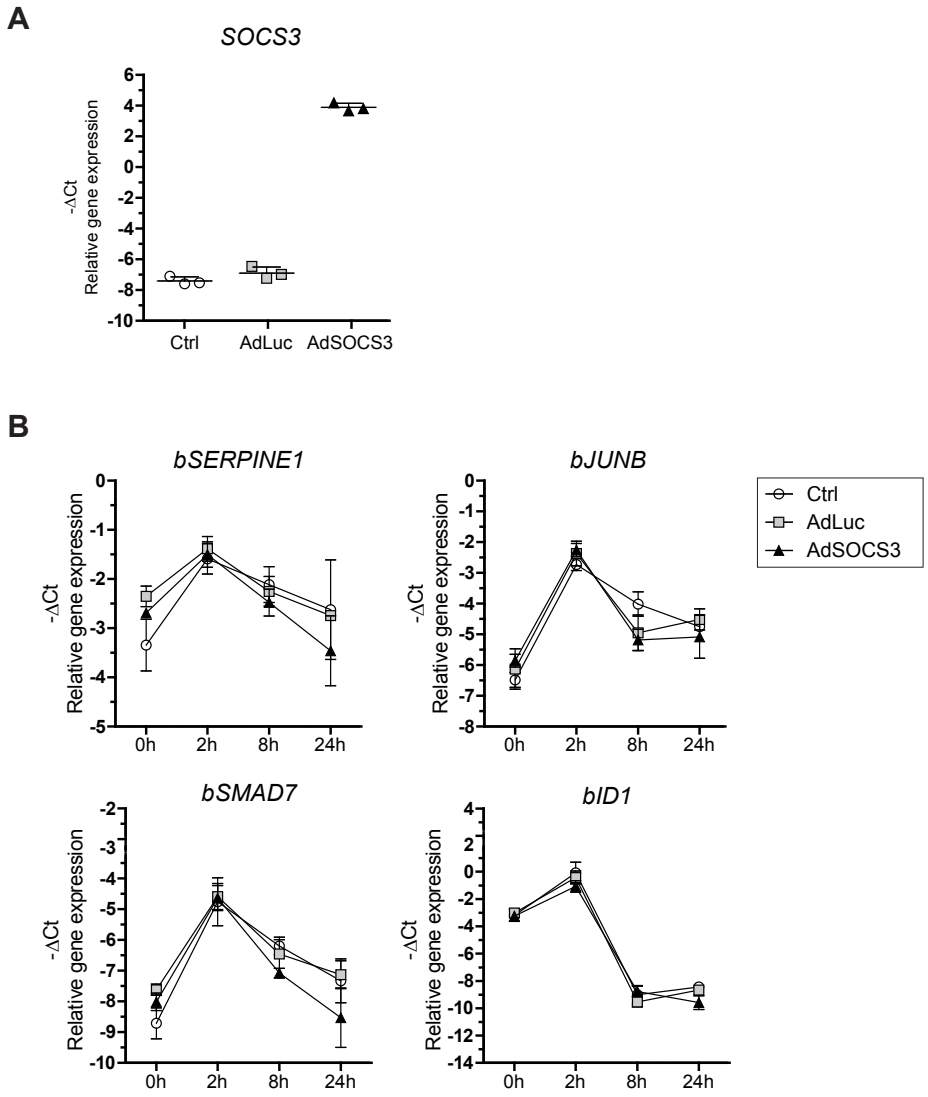
In conclusion, we show that OA-related inflammatory factors increase SOCS3 expression in articular chondrocytes. However, SOCS3 does not cause inflammation-mediated inhibition of TGF-β signaling in chondrocytes, as we found no evidence that SOCS3 inhibits TGF-β signaling on the level of receptor expression, p-Smad activation or TGF-β transcriptional activity. Strikingly, SOCS3 did strongly impair MSC-based cartilage formation. Based on our findings in chondrocytes, the effect of SOCS3 on cartilage formation is likely not caused by direct inhibition of TGF-β signaling, but works via a different mechanism. Thus, inflammation-induced SOCS3 may play a role in impaired MSC-based cartilage repair processes, which identifies SOCS3 as a potential target to improve chondrogenesis in the OA joint.

SUPPLEMENTARY DATA



Supplementary Figure 1. Overexpression of SOCS3 in primary bovine chondrocytes does not affect ALK1/ALK5 expression or activation of p-Smad2/3 versus p-Smad1/5/9 signaling. To study the effect of SOCS3 on TGF-β signaling, primary bovine chondrocytes were transduced with SOCS3- or luciferase adenovirus, or left untreated (control). **(A)** Gene expression of bALK1 and bALK5 receptors was analyzed 48 h after virus transduction using qPCR. Data represents mean ± SD of three technical replicates from one experiment **(B)** C-terminal phosphorylation of Smad2/3 or Smad1/5/9 was analyzed with western blot in primary bovine chondrocytes at 1 h after rhTGF-β stimulation (1.0 ng/ml). **(C)** SOCS3 protein expression in primary bovine chondrocytes as determined by Western blot. SOCS3 protein increased after

viral transduction with increasing multiplicity of infection (MOI) of SOCS3 adenovirus. Statistical differences in bALK1 and bALK5 expression were tested with Kruskall Wallis followed by Dunn's multiple comparison test.



Supplementary Figure 2. Overexpression of SOCS3 in primary bovine chondrocytes does not affect TGF-β-induced expression of Smad3-dependent genes. (A) To study the effect of SOCS3 on TGF-β-induced expression of Smad3-dependent genes, primary bovine chondrocytes were transduced with SOCS3- or luciferase adenovirus, or left untreated (no virus control). **(B)** After 48 h, bovine chondrocytes were stimulated with rhTGF-β for 2 h, 8h and 24 h and expression of bSERPINE1, bJUNB, bSMAD7, and bID1 was analyzed with qPCR. Data represents mean ± SD of three technical replicates from one experiment. Differences in TGF-β-mediated induction of bSERPINE1, bJUNB, bSMAD7 and bID1 were tested with Kruskall Wallis followed by Dunn's multiple comparison test.

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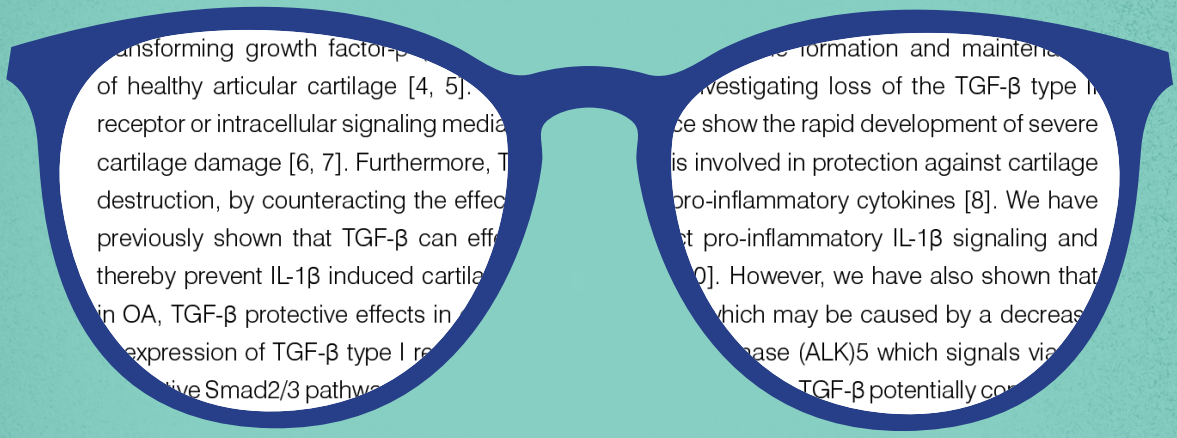

Chapter 3

TGF-β dampens IL-6 signaling in articular chondrocytes by decreasing IL-6 receptor expression

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ABSTRACT

Objective

Transforming growth factor-β (TGF-β) is an important homeostatic regulator of cartilage. In contrast, interleukin-6 (IL-6) is a pro-inflammatory cytokine implicated in cartilage degeneration. Cross-talk between TGF-β and IL-6 is reported in tissues other than articular cartilage. Here, we investigated regulation of IL-6 signaling by TGF-β in articular chondrocytes.

Design

Human primary chondrocytes and the human G6 chondrocyte cell line were stimulated with TGF-β1 or interleukin-1β (IL-1β). Expression of IL-6 and IL-6 receptor (IL-6R) was determined on mRNA and protein level. TGF-β regulation of IL-6 signaling via phospho-STAT3 (p-STAT3) was determined using Western blot, in presence of inhibitors for IL-6R, and Janus kinase (JAK)- and activin receptor-like kinase (ALK)5 activity. Furthermore, induction of STAT3-responsive genes was used as a read-out for IL-6-induced gene expression.

Results

TGF-β1 increased IL-6 mRNA and protein expression in both G6 and primary chondrocytes. Moreover, TGF-β1 stimulation clearly induced p-STAT3, which was abolished by inhibition of either IL-6R, JAK- or ALK5 kinase activity. However, TGF-β1 did not increase expression of the STAT3-responsive gene SOCS3 and pre-treatment with TGF-β1 even inhibited induction of p-STAT3 and SOCS3 by rhIL-6. Interestingly, TGF-β1 potently decreased IL-6R expression. In contrast, IL-1β did increase IL-6 levels, but did not affect IL-6R expression. Finally, addition of recombinant IL-6R abolished the inhibitory effect of TGF-β1 on IL-6-induced p-STAT3 and downstream *SOCS3*, *BCL3*, *SAA1* and *MMP1* expression.

Conclusions

In this study we show that TGF-β decreases IL-6R expression, thereby dampening IL-6 signaling in chondrocytes. This reveals a novel effect of TGF-β, possibly important to restrict pro-inflammatory IL-6 effects to preserve cartilage homeostasis.

Introduction

The main function of chondrocytes is to maintain cartilage homeostasis, which is a balance between cartilage matrix synthesis and degradation. This balance is tightly regulated by soluble anabolic and catabolic factors. During osteoarthritis (OA), the balance shifts towards catabolic processes, leading to cartilage damage. In OA cartilage, chondrocytes display a catabolic phenotype and produce matrix degrading enzymes such as matrix metalloproteinase (MMP)-13, leading to destruction of surrounding cartilage matrix [1-3]. It is unclear why OA chondrocytes display this catabolic phenotype, but both increased levels of catabolic mediators and a changed response to anabolic factors are thought to contribute to this process.

Transforming growth factor-β (TGF-β) is a crucial factor for the formation and maintenance of healthy articular cartilage [4, 5]. Knock-out studies investigating loss of the TGF-β type II receptor or intracellular signaling mediator Smad3 in mice show the rapid development of severe cartilage damage [6, 7]. Furthermore, TGF-β signaling is involved in protection against cartilage destruction, by counteracting the effects of catabolic, pro-inflammatory cytokines [8]. We have previously shown that TGF-β can effectively counteract pro-inflammatory IL-1β signaling and thereby prevent IL-1β induced cartilage destruction [9, 10]. However, we have also shown that in OA, TGF-β protective effects in cartilage are impaired, which may be caused by a decrease in expression of TGF-β type I receptor activin receptor like-kinase (ALK)5 which signals via the protective Smad2/3 pathway [11, 12]. This loss in responsiveness to TGF-β potentially contributes to the imbalance in cartilage homeostasis observed in OA.

Interleukin-6 (IL-6) is considered to be a catabolic factor for chondrocytes because several studies show a role for IL-6 in cartilage degradation [13-15]. IL-6 is present in increased levels in serum and synovial fluid of OA patients compared to unaffected individuals, and circulating IL-6 levels are even considered to be predictive for OA development [16, 17]. *In vitro*, IL-6 enhances the expression of MMP-3 and -13 in bovine chondrocytes, as well as the expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 and -5 [13]. Also in mice, intra-articular injection of IL-6 results in cartilage damage and increased production of MMP-3 and -13 [14]. Moreover, therapeutically blocking IL-6 or its downstream mediator STAT3 during experimental OA evidently rescues cartilage damage and osteophyte formation [15].

Interestingly, regulation of IL-6 signaling by TGF-β has been reported in various cell types [18-21]. For example, TGF-β blocks IL-6-mediated phosphorylation of intracellular mediators STAT1 and STAT3 in intestinal epithelial cells, as well as the induction of ICAM expression [18]. Furthermore, TGF-β can induce the expression of suppressor of cytokine signaling (SOCS)3, a negative regulator of the IL-6 pathway, in macrophage/osteoclast precursors, but not in T-cells [20, 22]. In contrast, TGF-β synergizes with IL-6 in T-cells by promoting the degradation of FOXP3

[19]. This shows that TGF-β regulation of IL-6 signaling is context- and cell type dependent. Although it is evident that TGF-β and IL-6 play important roles in regulating cartilage homeostasis and degeneration, a link between these two factors in chondrocytes remains to be identified. Therefore, the goal of this study was to investigate potential regulation of IL-6 signaling by TGF-β in articular chondrocytes. This will improve insights in the functional role of TGF-β in cartilage homeostasis.

MATERIALS AND METHODS

Patient material

Primary human chondrocytes were isolated from macroscopically intact cartilage obtained after surgery from seven anonymized OA patients undergoing total knee or hip arthroplasty (Radboud University Medical Center, Nijmegen, the Netherlands). Patients were informed about the anonymized use of this material for research and were able to refuse. According to Dutch law, informed consent is therefore not necessary.

Primary cell culture

To obtain chondrocytes, cartilage explants were digested overnight in 1.5 mg/ml Collagenase B (Roche Diagnostics) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco). Chondrocytes were seeded in a density of $1x10^5$ cells/cm^2 in 6-well plates (Cellstar; Greiner Bio-one International) for protein experiments, or in 24-wells plates (Cellstar; Greiner Bio-one International) for gene expression experiments. Chondrocytes were cultured in monolayer for one week prior to experiments in DMEM/F12 supplemented with 10% fetal calf serum (FCS; Thermo Scientific), 100 mg/l pyruvate (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin in standard conditions [5% CO2 (v/v), 37 °C, 95% humidity]. Chondrocytes were serum-starved overnight in DMEM/F12 medium without FCS before start of the experiment.

Chondrocyte cell line culture

Human G6 chondrocytes are adult articular chondrocytes derived from femoral head cartilage of an anonymous OA donor, transduced with a temperature-dependent SV40 large oncogene [23]. Consequently, the G6 chondrocytes proliferate at 32°C, but not at 37°C. G6 chondrocytes were cultured at 32°C under the same conditions as primary chondrocytes, except with 5% FCS. Two days before experiments were performed, G6 chondrocytes were cultured at 37°C to stop proliferation and serum-starved overnight in DMEM/F12 medium supplemented with 0.5% FCS.

Culture with cytokines or pharmacological inhibitors

Serum starved primary chondrocytes and G6 chondrocytes were stimulated with rhTGF-β1 (Biolegend), rhIL-1β (R&D systems), rhIL-6 (Biolegend), rhIL-6R (Biolegend) or a combination of

these proteins, for time periods and dosages indicated in figure legends. In experiments where inhibitors were used, dimethylsulfoxide (DMSO) (0.05% (v/v)) was used as vehicle control. To inhibit ALK5 kinase activity, SB-505124 (Sigma Aldrich) [24] was used in a concentration of 5 μM. For inhibition of Janus kinases (JAKs), the pan-JAK inhibitor Tofacitinib (LC Laboratories) was used in a concentration of 1 μM. A humanized anti-IL-6R monoclonal antibody (Tocilizumab, RoActemra, Roche) was used to block IL-6 signaling at 1 μg/ml. Chondrocytes were pre-incubated with the inhibitors 1 h prior to addition of rhTGF-β1.

Protein measurement using Luminex technology

Luminex multianalyte technology on the Bio-Plex 200 system (Bio-Rad) in combination with multiplex cytokine kit (Bio-Rad) was used to study levels of IL-6 in 50 ml of chondrocyte culture supernatants after rhTGF-β1 stimulation. Samples below detection limit (0.40 pg/ml) were set at lowest measurable concentration to perform statistical analysis.

Protein isolation and Western blot

Chondrocytes were lysed using lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Roche Diagnostics). Cell lysates were sonicated on ice using a Bioruptor (Diagenode) [25]. Protein concentration was normalized between samples using a bicinchoninic acid assay (Thermo Scientific). Samples were boiled for 10 min at 95°C in Laemmli Sample buffer (1% SDS and 100mM Tris, pH 9.5). Proteins were separated on a 10% reducing bis-acrylamide SDS-PAGE gel, and transferred to a 0.45 μm pore nitrocellulose membrane using wet transfer (Towbin buffer, 2h, 275 mA at 4°C). Non-specific antibody binding was blocked for 1 h with 5% non-fat dry milk (Campina) to detect IL-6R, β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or blocked in 5% bovine albumin serum to detect phosho-STAT3 (p-STAT3) or phospho-Smad2/3 (p-Smad2/3), in TBS-T (15 mM Tris-HCl, pH 7.4, 0.1% Tween). Membranes were incubated overnight at 4°C with primary antibodies directed against IL-6R (polyclonal rabbit antibody, 128008, 1:1000, Abcam) , p-STAT3 (polyclonal rabbit antibody, #9131, 1:1000, Cell Signaling), or p-Smad2/3 (polyclonal rabbit antibody, #3101, 1:1000, Cell Signaling) and afterwards labeled for 1 h with polyclonal Goat anti-Rabbit or Rabbit anti-Mouse coupled to horseradish peroxidase (1:1500, Dako) at RT. Enhanced chemiluminescence (ECL) was used to visualize proteins with ECL prime kit (GE Healthcare) and ImageQuant LAS4000 (Leica). GAPDH was used as loading control (mouse monoclonal antibody, 1G5, 1:10,000 Sigma Aldrich). ImageJ was used for quantification of the signal.

RNA isolation and quantitative real-time PCR

RNA was isolated using Tri-reagent (Sigma-Aldrich) following manufacturer's protocol. RNA concentrations were determined using a NanoDrop photospectrometer (Thermo Scientific). Thereafter, a maximum of 1 μg RNA was dissolved in ultrapure water and treated with 1 μl of DNAse (Life Technologies) for 15 min at room temperature to remove possible genomic

DNA, followed by incubation at 65°C with 1 µl EDTA (Life Technologies). Samples were reverse transcribed into complementary DNA (cDNA) using 1.9 µl ultrapure water, 2.4 µl 10x DNase buffer, 2.0 µl 0.1 M dithiothreitol, 0.8 µl 25 mM dNTPs, 0.4 µg oligo dT primer, 1 µl 200 U/ml MMLv-reverse transcriptase (Life Technologies) and 0.5 µl 40U/ml RNAsin (Promega) and incubated for 5 min at 25 °C, 60 min at 39 °C, and 5 min at 95 °C using a thermo-cycler. Gene expression was measured using SYBR Green Master Mix (Applied Biosystems) and 0.25 mM primers (see Table 1) (Biolegio) with a StepOnePlus real-time PCR system (Applied Biosystems). The amplification protocol was 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were analyzed to confirm product specificity. To calculate the relative gene expression, the average of the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein S27A (RPS27A) was used.

Table 1. Template and sequence of the primers used in this study.

Gene	Forward Sequence (5' -> 3')	Reverse Sequence (5' -> 3')
GAPDH	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTGAGC
IL6	AGCCCACCGGGAACGA	GGACCGAAGGCGCTTGT
IL6R	GTACCACTGCCCACATTCCT	CCACGTCTTCTTGAACCTCAGA
RPS27a	GTTAAGCTGGCTGTCCTGAAA	CATCAGAAGGGCACTCTCG
SOCS3	TCGGACCAGCGCCACTT	CACTGGATGCGCAGGTTCT
SAA1	GTGATCAGCGATGCCAGAGA	TCGGAAGTGATTGGGGTCTT
BCL3	GGAGGCCCGCAATTATGA	CTTAATGTCCACTGCGTCGAT
MMP1	ACTGCCAAATGGGCTTGAAG	TTCCCTTTGAAAAACCGGACTT

Statistical analysis

Quantitative data of gene expression analysis was expressed as column scatter or grouped category graphs and display mean values of separate experiments (G6 chondrocytes) or donors (primary chondrocytes) with corresponding 95% confidence interval (CI). Significance was tested using displayed means with repeated measures ANOVA followed by Dunnett's or Bonferroni post-test (see figure legends). Differences in IL-6 protein levels after rhTGF-β1 stimulation were tested using an unpaired two-tailed t-test. For each analysis, Gaussian distribution was tested using the D'Agostino-Pearson Omnibus K2 test. $P < 0.05$ was considered significant. All analyses were performed using Graph Pad Prism version 5.03 (GraphPad Software, La Jolla, CA).

RESULTS

TGF-β1 rapidly induces IL-6 in human chondrocytes

To examine if TGF-β1 can regulate IL-6 expression, G6 chondrocytes were stimulated with 1.0 ng/ml of TGF-β1. TGF-β1 significantly induced *IL6* mRNA expression after 2 h, 4 h and 6 h by respectively 21-, 38- and 112-fold (4.4, 5.3, 6.8 ΔC_t) (Fig. 1A). To validate these results, we performed the same experiment in freshly isolated primary chondrocytes of four different donors. Although variation between donors was observed, TGF-β1 induced a significant increase in *IL6* expression of on average 32-fold (5.0 ΔC_t) at 4 h and 74-fold (6.2 ΔC_t) at 6 h (Fig. 1B). Similar induction of *IL6* expression was observed when 0.1 ng/ml of rhTGF-β1 was used, corresponding to TGF-β levels found in synovial fluid of healthy donors (Supplementary Fig. 1A) [26], We further confirmed these results on protein level, and observed a significant 20-fold increase in IL-6 production after TGF-β1 stimulation of G6 chondrocytes (Fig. 1C).

TGF-β1 induces phosphorylation of STAT3 *via* IL-6 receptor signaling

To investigate if TGF-β1-induced IL-6 leads to autocrine signaling, we investigated the pattern of p-STAT3, an IL-6R downstream signaling protein. TGF-β1 stimulation of G6 chondrocytes induced a clear increase in p-STAT3 at 2 h and 4 h (Fig. 2A). In contrast, total levels of STAT3 protein were not changed by stimulation with rhTGF-β1. When we studied the p-STAT3 pattern in primary chondrocytes from different donors, we found that p-STAT3 was consistently upregulated by TGF-β1 at 2 h and 4 h in all donors. In two out of four donors, we observed a minor increase at 5-15 min, but this was not consistent throughout experiments. Because G6 chondrocytes and primary chondrocytes showed similar patterns of p-STAT3 induction by TGF-β1, we used G6 chondrocytes for further inhibitor studies. To test if TGF-β1-induced p-STAT3 was IL-6R dependent, we stimulated G6 chondrocytes with TGF-β1 in presence of the IL-6R blocking antibody tocilizumab. As expected, induction of p-STAT3 by TGF-β1 was completely prevented by blockade of the IL-6R suggesting IL-6 dependency (Fig. 2B). To further explore which signaling proteins are responsible for induction of IL-6 and p-STAT3 via TGF-β1 we used small molecule inhibitors. Inhibition of TGF-β receptor (ALK5) kinase activity with SB-505124 inhibited both induction of p-Smad2/3 and p-STAT3 at all time points after TGF-β1 stimulation (Fig. 2C). Furthermore, addition of the pan-JAK inhibitor tofacitinib completely prevented induction of p-STAT3 by TGF-β1 and even decreased baseline levels (Fig. 2D). This effect was not due to inhibition of the TGF-β signaling pathway, as the increase in p-Smad2/3 by TGF-β1 was not inhibited by tofacitinib. Altogether, this shows that TGF-β1-induced p-STAT3 depends on the IL-6R, and ALK5- and JAK-kinase activity.

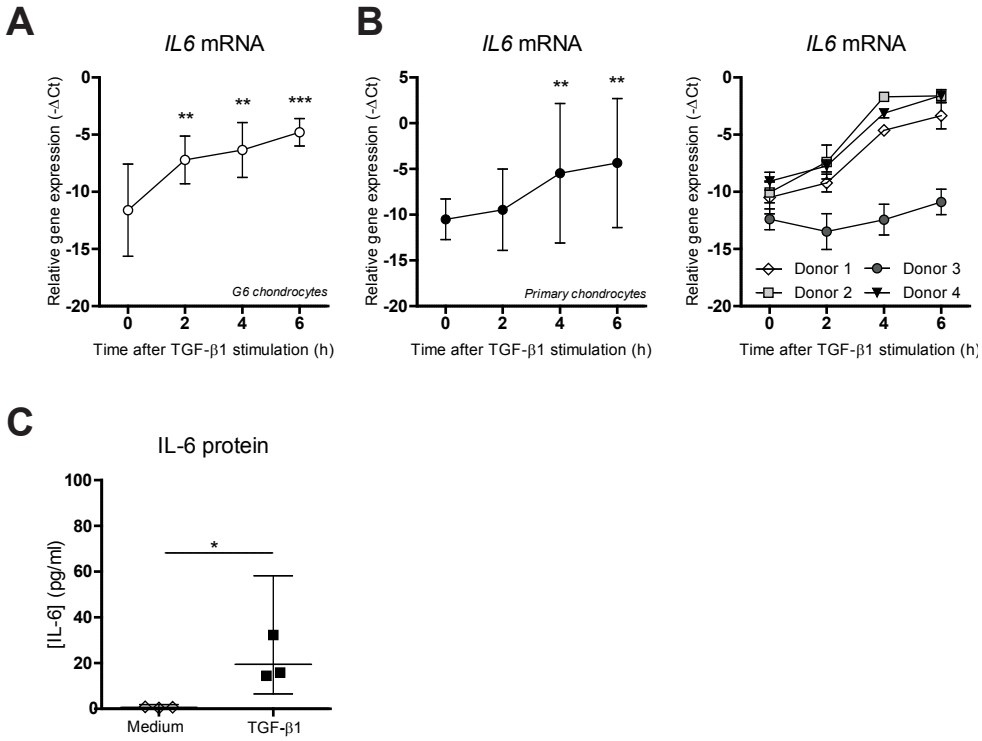


Figure 1. TGF-β1 stimulation of human chondrocytes rapidly induces IL-6. The human G6 chondrocyte cell line and primary human chondrocytes of four donors were stimulated in triplo with 1.0 ng/ml of rhTGF-β1 for 2, 4 and 6 hours to study TGF-β1-induced IL6 gene expression using qPCR. **(A)** For G6 chondrocytes, the mean of three separate experimental repeats is shown with corresponding 95% confidence interval (CI). **(B)** For primary chondrocytes, the mean of four donors is shown with corresponding 95% CI, and individual donors are plotted showing mean ± SD of technical replicates **(C)** G6 chondrocytes were stimulated with 1.0 ng/ml of rhTGF-β1 for 24 hours, after which the concentration of IL-6 in the supernatant was measured using Luminex technology. The means of three separate experiments are shown with corresponding 95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by repeated measures ANOVA with Dunnett's post-test (**A, B)** or unpaired two-tailed t-test **(C)**.

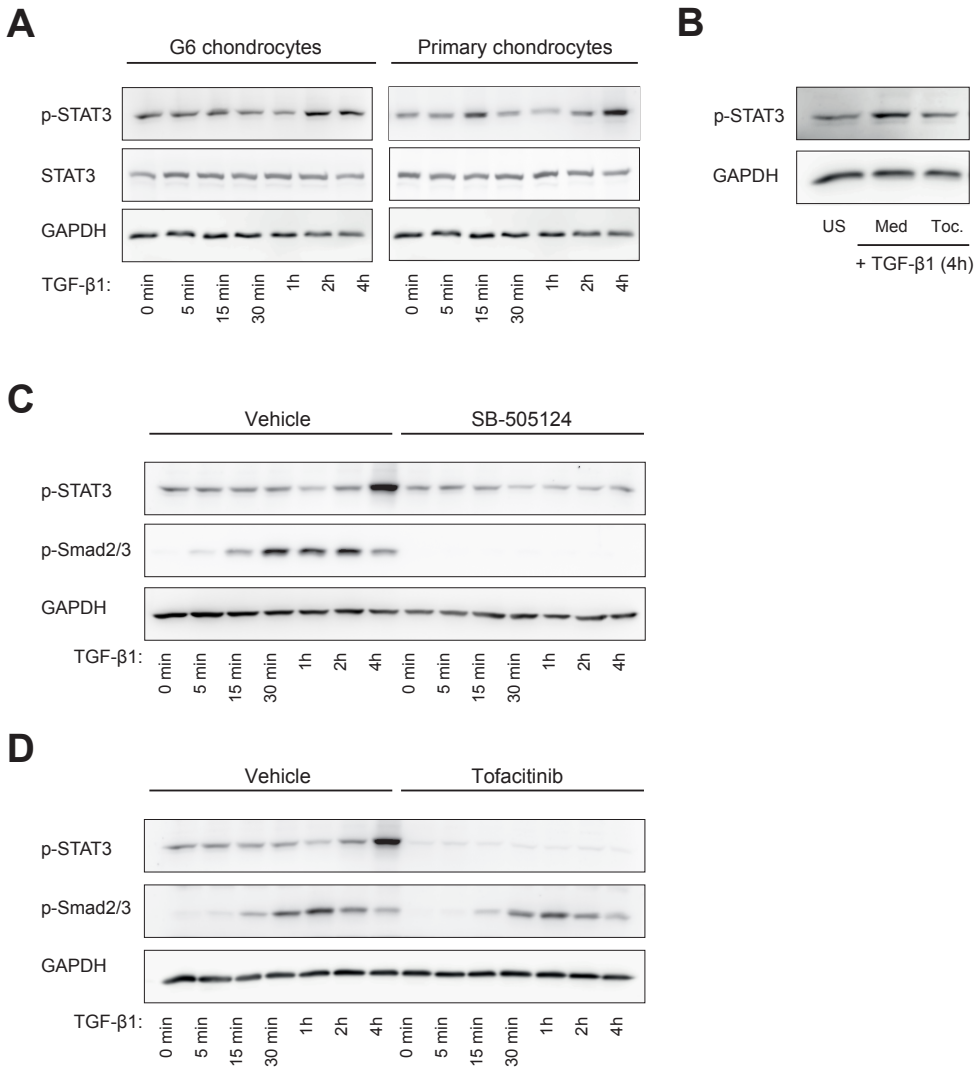


Figure 2. TGF-β1 stimulation leads to STAT3 phosphorylation, dependent on the IL-6R, as well as ALK5- and JAK-kinase activity. (A) The human G6 chondrocyte cell line and primary human chondrocytes of three donors were stimulated with 1.0 ng/ml of rhTGF-β1 for 5, 15, or 30 min and 1, 2, and 4 h, to study TGF-β1-induced STAT3 phosphorylation with Western Blot. **(B)** Tocilizumab (anti-IL-6R antibody) was used to investigate if TGF-β1-induced STAT3 phosphorylation was dependent on IL-6R signaling. G6 chondrocytes were pre-incubated with 1 μg/ml of Tocilizumab for 1 h, and afterwards stimulated with 1 ng/ml of rhTGF-β1 for 4 h. Small molecule inhibitors for **(C)** TGF-β receptor kinase activity (SB-505124, 5 μM) and **(D)** JAK-kinase activity (Tofacitanib, 1 μM) were used to investigate if TGF-β1-induced STAT3 phosphorylation was dependent on ALK5- or JAK kinase activity respectively. G6 chondrocytes were pre-incubated with inhibitors or vehicle for 1 h, and stimulated with 1 ng/ml of rhTGF-β1 for indicated time points. Western Blots shown are representative of at least three independent experiments. Toc: Tocilizumab; Med: medium; US: unstimulated.

TGF-β1 blocks IL-6-mediated gene expression of *SOCS3* and limits STAT3 phosphorylation

We showed that TGF-β1 induces IL-6 signal transduction via activation of its downstream mediator STAT3. To investigate if the observed STAT3 phosphorylation results in gene transcription, mRNA expression of the well-known STAT3 target gene *SOCS3* was measured, which itself is an important negative regulator of IL-6 signaling [27]. Because *SOCS3* expression is immediately induced after p-STAT3 induction [28], we investigated the effect of TGF-β1 on *SOCS3* expression at 6 h, shortly after TGF-β1 induction of p-STAT3 (2-4 h). Surprisingly, we did not find any evidence of an increase in *SOCS3* mRNA expression by TGF-β1 (Fig. 3A). Also addition of tocilizumab, which blocks TGF-β1-induced IL-6 signaling, did not significantly change *SOCS3* expression. In contrast, stimulation of G6 chondrocytes with 10 ng/ml of recombinant IL-6 did lead to a significant increase in *SOCS3* expression of 3.0-fold (1.6 ΔC_t), confirming that *SOCS3* is an IL-6/STAT3 target gene in our experimental setting (Fig. 3A). Thus, stimulation with IL-6 alone increased *SOCS3* expression, but stimulation with TGF-β1 did not, while this ultimately also leads to IL-6 production. This suggests that TGF-β1 blocks IL-6-induced regulation of *SOCS3* expression. To further prove that TGF-β1 indeed inhibits IL-6 effects, we investigated if TGF-β1 could also block the effects of recombinant IL-6. We performed pre-treatment with TGF-β1 for a 6 h period, mimicking the setting where cells are first exposed to TGF-β1 and afterwards to IL-6. Interestingly, activation of STAT3 after rhIL-6 exposure was inhibited by TGF-β1 pre-treatment, but p-STAT3 levels were not fully reduced to baseline level (Fig. 3B). Furthermore, TGF-β1 pre-treatment completely prevented rhIL-6-induced *SOCS3* expression (Fig. 3C). These data demonstrate that TGF-β1 inhibits the IL-6 response in chondrocytes, despite upregulating IL-6 itself.

TGF-β1 potently decreases IL-6 receptor levels in articular chondrocytes

Next, we studied the effects of TGF-β1 on the IL-6R, a potential mechanism via which TGF-β could regulate IL-6 intracellular signaling. Interestingly, we observed that TGF-β1 stimulation led to a striking decrease in *IL6R* expression in G6 chondrocytes after 4 h (13-fold, 3.7 ΔC_t) and 6 h (8-fold, 3.0 ΔC_t) (Fig. 4A). This effect of TGF-β1 also held true in primary chondrocytes derived from different donors, showing that the effect of TGF-β on *IL6R* expression is not donor specific. In primary chondrocytes, TGF-β1 significantly decreased *IL6R* expression after both 4 h and 6 h with an average of 2.75-fold (1.5 ΔC_t) and 3.2-fold (1.7 ΔC_t) respectively (Fig. 4B). In addition, 0.1 ng/ml of rhTGF-β1, a concentration reflecting TGF-β levels in synovial fluid of healthy donors [26], decreased *IL6R* expression to the same extent (Supplementary Fig. 1B). To investigate if other factors stimulating IL-6 production can also regulate *IL6R* expression, we included stimulation with the pro-inflammatory cytokine IL-1β, a known inducer of IL-6. As expected, IL-1β did strongly elevate *IL6* expression by 1024-fold (10.0 ΔC_t) after 4-6 h, even to a higher extent than TGF-β1 (Fig. 4C). Interestingly, in contrast to TGF-β1, IL-1β did not significantly change the expression of *IL6R* in G6 chondrocytes. This suggests that IL-6 itself is not involved in

regulation of *IL6R* expression. Also on protein level, we observed significantly lower IL-6R levels after 24 h of TGF-β1 stimulation of G6 chondrocytes, while IL1β even increased IL-6R levels (Fig. 4D). These data show that TGF-β1 decreases IL-6R expression, which could explain why TGF-β1 inhibits IL-6 signaling.

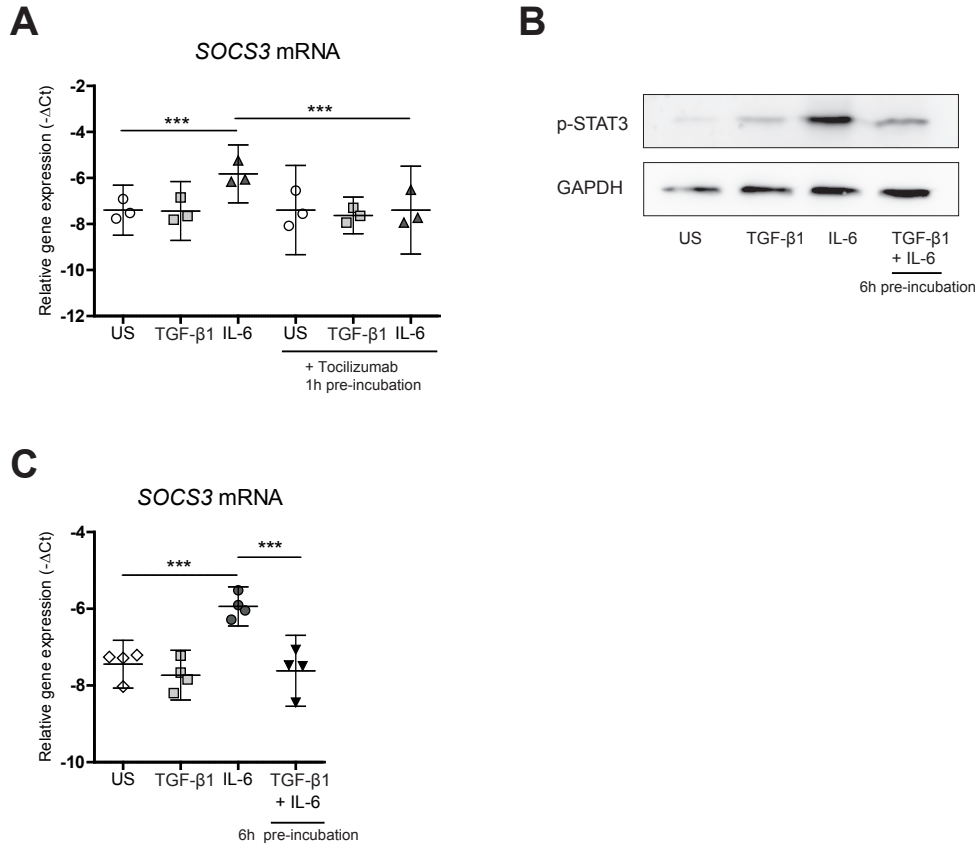


Figure 3. TGF-β1 blocks IL-6 mediated gene expression of SOCS3 and limits STAT3 phosphorylation. **(A)** To investigate the downstream effects of TGF-β1-induced IL-6 signaling, G6 chondrocytes were pre-incubated with 1 μg/ml of Tocilizumab to block IL-6R signaling, and stimulated with TGF-β1 for 6 h. Expression level of the STAT3 target gene SOCS3 was measured as a read-out for IL-6-mediated gene expression. **(B, C)** To study the effect of TGF-β1 on signaling of exogenous rhIL-6, human G6 chondrocytes were pre-stimulated with or without 1.0 ng/ml of rhTGF-β1 for 6 hours, and afterwards stimulated with rhIL-6 (10 ng/ml). The IL-6 response was measured by induction of STAT3 phosphorylation after 30 min **(B)** or induction of SOCS3 mRNA after 6 h **(C)**. For Western Blot is representative of three independent experiments. For gene expression results, the means of three or four separate experimental repeates are plotted with corresponding ± 95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by repeated measurements ANOVA with Bonferroni's post-test. US: unstimulated.

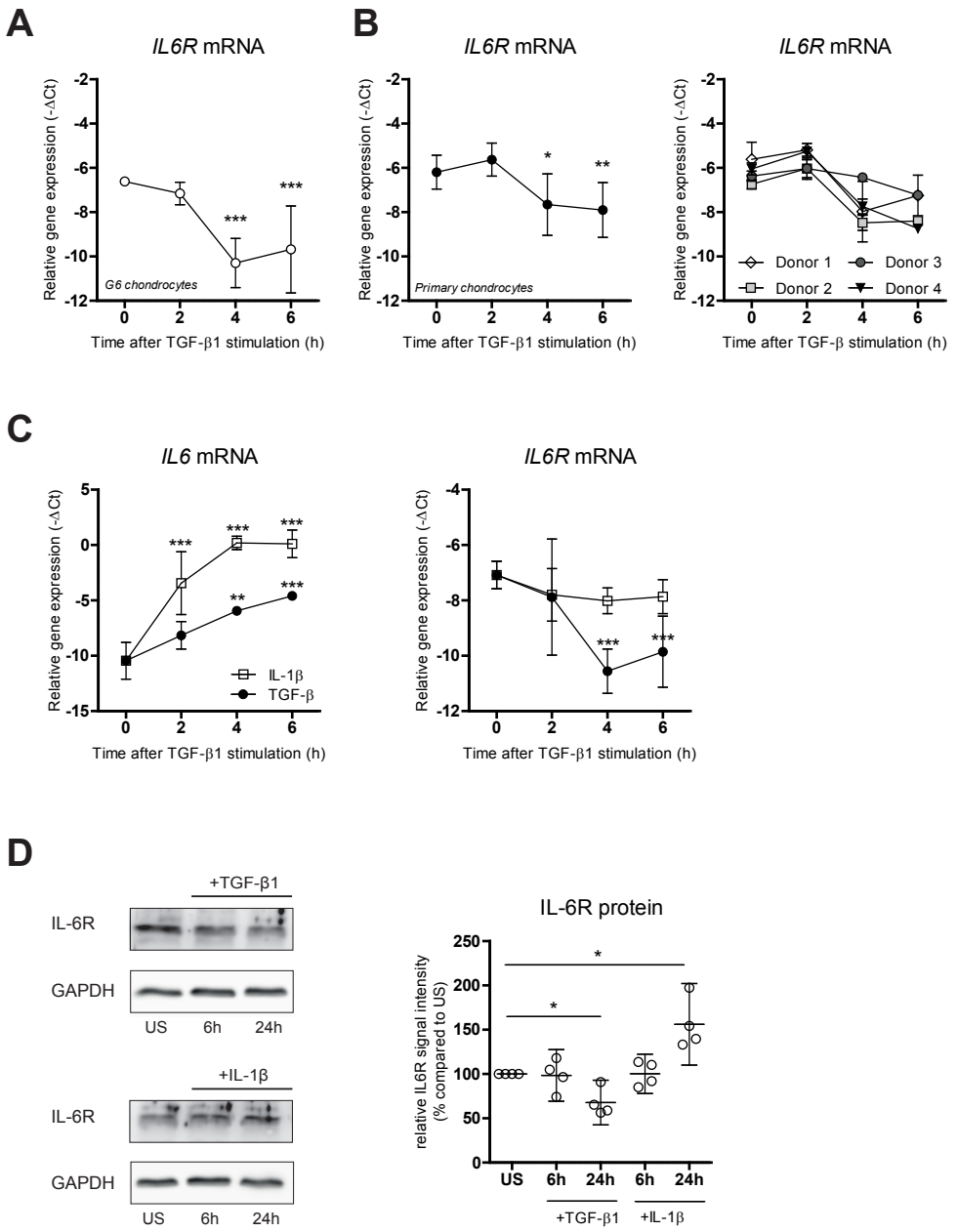


Figure 4. TGF-β1 potently decreases IL-6 receptor levels in both G6 and primary chondrocytes. To investigate the effect of TGF-β1 on expression of the *IL6R*, G6 chondrocytes and primary human chondrocytes of four donors were stimulated in triplicate with 1.0 ng/ml of rhTGF-β1 for 2, 4 or 6 hours. **(A)** For G6 chondrocytes, the mean of three separate experimental repeats is shown with corresponding 95% CI. **(B)** For primary chondrocytes, the mean of four donors is shown with corresponding 95% CI, and individual

donors are plotted showing mean ± SD of technical replicates. **(C)** G6 chondrocytes were stimulated with 1.0 ng/ml of rhTGF-β1 or 1.0 ng/ml of rhIL-1β for 2, 4 or 6 hours to study effects on *IL6* and *IL6R* expression. Mean of four separate experimental repeats is shown with corresponding 95% CI. **(D)** G6 chondrocytes were stimulated with 1.0 ng/ml of rhTGF-β1 or 1.0 ng/ml of rhIL-1β for 6 h and 24 h to determine effects on IL-6R protein expression using Western Blot. Western blots are representative of four independent experiments. Quantification of the Western Blot was performed by Image J. Quantitative values of IL-6R protein intensity were first corrected for GAPDH values, and then plotted as fold change (%) compared to US samples. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by one-way ANOVA with Dunnett's **(A, B, C)**or Bonferroni **(D)** post-test. US: unstimulated.

TGF-β1 blocks the IL-6 response by decreasing IL-6 receptor levels

To determine the consequences of TGF-β1-mediated down regulation of IL-6R expression, we investigated if TGF-β1-mediated inhibition of IL-6 signaling was dependent on reduced IL-6R levels. We performed pre-treatment with TGF-β1 for a 6 h period, to ensure that IL-6R levels were decreased before stimulation with rhIL-6. In addition, we rescued IL-6R levels using a soluble form of the IL-6R. Strikingly, levels of p-STAT3 were dose-dependently increased after adding soluble IL-6R, showing that TGF-β1 inhibition of rhIL-6-induced p-STAT3 is dependent on the IL-6R (Fig. 5A). On gene expression level, the addition of rhIL-6R also restored induction of *SOCS3* expression by rhIL-6 in the presence of TGF-β1 (Fig. 5B). Moreover, the expression of additional IL-6-responsive genes, *MMP1, BCL3* and *SAA1*, was comparably regulated. Pre-treatment with rhTGF-β1 clearly inhibited the induction of these genes by rhIL-6, which was abolished by addition of rhIL-6R. Altogether, this indicates that TGF-β1 dampens IL-6 effects on chondrocytes by decreasing IL-6R expression (Fig. 6).

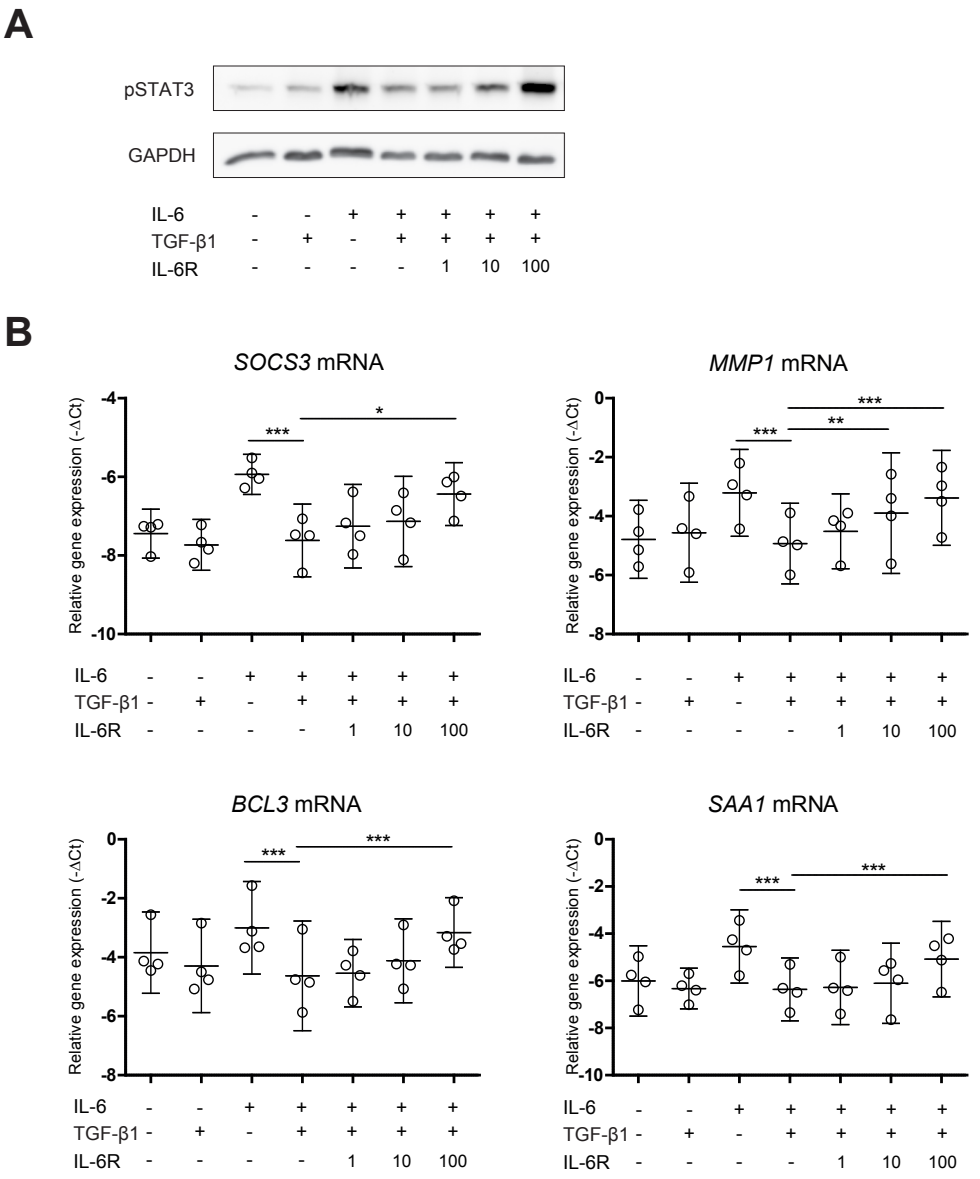


Figure 5. TGF-β1-mediated inhibition of IL-6 signaling is dependent on decreased IL-6R levels. To study the effect of TGF-β1 on IL-6 signaling with or without recombinant IL-6R, human G6 chondrocytes were pre-stimulated with 1.0 ng/ml of rhTGF-β1 for 6 h, and afterwards stimulated with rhIL-6 (10 ng/ml) alone or in combination with rhIL-6R (1, 10 and 100 ng/ml). The IL-6 response was measured by induction of p-STAT3 after 30min of IL-6 stimulation **(A)** or induction of *SOCS3*, *MMP1*, *BCL3* and *SAA1* expression after 6 h **(B)**. Western blots are representative of three independent experiments. For gene expression studies, the means of four separate experimental repeates are plotted with corresponding ± 95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by repeated measures ANOVA with with Bonferroni's post-test.

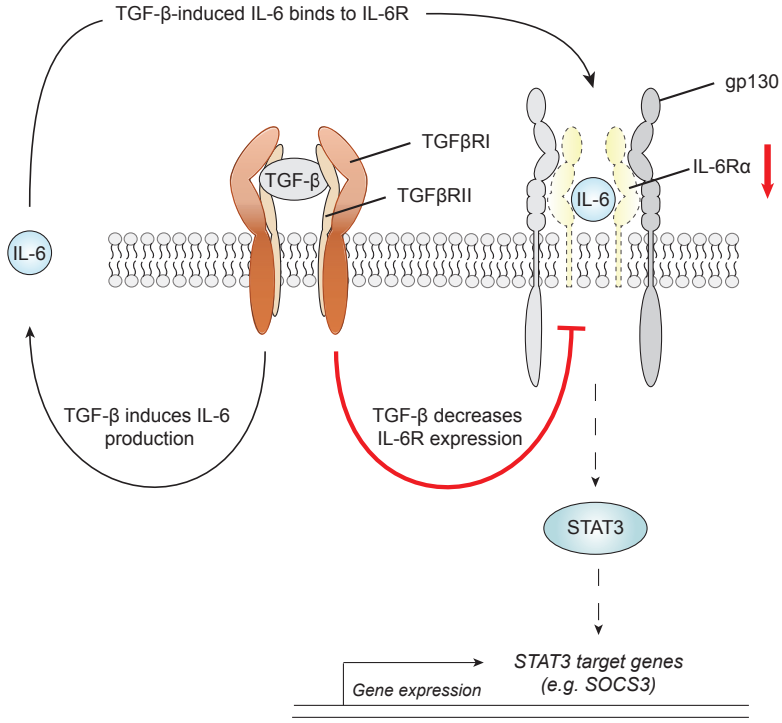


Figure 6. Schematic overview of TGF-β1-mediated regulation of IL-6 signaling in chondrocytes. TGF-β1 induces the release of IL-6 in human articular chondrocytes. Released IL-6 binds in an autocrine manner to membrane-bound IL-6 receptor on chondrocytes and leads to phosphorylation of the intracellular signaling mediator STAT3. Simultaneously, TGF-β1 decreases IL-6 receptor expression on chondrocytes, resulting in limited STAT3 phosphorylation and inhibition of STAT3-responsive target genes.

DISCUSSION

In this study we identified TGF-β as a key regulator of the IL-6 signaling pathway in articular chondrocytes. We show that articular chondrocytes can produce IL-6 in response to TGF-β1, but that downstream signaling is limited. We show that this inhibition is facilitated by down regulation of the IL-6R by TGF-β1, a unique mechanism which had never been reported until now.

IL-6 is a pleiotropic cytokine that has an essential role in regulating immune response and defense [29]. In OA, levels of IL-6 are enhanced in serum and the synovial fluid and correlate with disease progression [16, 17]. Within the joint, both synoviocytes and chondrocytes can produce IL-6 and several inflammatory factors are known to increase IL-6 production [30-32]. We show that also stimulation with TGF-β1, a homeostatic regulator of cartilage, leads to IL-6 production in chondrocytes. Only Guerner et al. previously studied regulation of IL-6 production in chondrocytes in response to both catabolic and anabolic factors. They reported that TGF-β, but not epidermal-, insulin-, or platelet derived-growth factor, increased the production of IL-6 by chondrocytes [33]. This observation was surprising, because TGF-β is perceived as a homeostatic regulator of cartilage [8]. In contrast, IL-6 is mainly recognized as a catabolic mediator that activates MMPs and ADAMTSs enzymes, contributing to cartilage degradation [14, 15, 34, 35]. Interestingly, we discovered that IL-6 induced by TGF-β1 did not lead to downstream induction of the STAT3 target gene *SOCS3*. Furthermore, TGF-β1 blocked the effects of recombinant IL-6, by limiting STAT3 phosphorylation and blocking induction of SOCS3. Together, this suggests that the presence of TGF-β can block downstream IL-6 signaling and thereby protect chondrocytes from the catabolic effects of IL-6.

Inhibition of IL-6 signaling by TGF-β had not yet been reported in articular chondrocytes. Also in other cell types only few studies investigated inhibitory effects of TGF-β on IL-6 signaling. In T-cells it has been shown that TGF-β inhibits IL-6-induced *SOCS3* expression, thereby promoting Th17 development [20]. However, TGF-β did not reduce IL-6-mediated p-STAT3, in contrast to our results. This implies that in T-cells there might be specific inhibition of SOCS3, and not a general inhibition of IL-6 effects. In intestinal epithelial cells, TGF-β inhibits IL-6 signaling in a similar manner as we observed in chondrocytes [18]. Pre-treatment with TGF-β inhibited IL-6-mediated induction of both p-STAT1 and p-STAT3, as well as IL-6-induced gene expression of ICAM [18]. Until now it was unclear via which mechanism TGF-β blocked IL-6 signaling.

In this study, we demonstrate that TGF-β1 can block IL-6 signaling *via* decreasing IL-6R levels. Because cartilage contains large amounts of inactive TGF-β, which can be activated upon loading, TGF-β-mediated down regulation of IL-6R could be involved in homeostatic regulation of cartilage [4, 11]. This is an entirely new concept, as there is no literature reporting that TGF-β decreases IL-6R levels in cartilage. This effect of TGF-β might be independent of IL-6, as we showed that IL-1β increased IL-6 production but did not affect IL-6R expression. In literature, inflammatory factors such as LPS and IL-1β are mainly known to increase *IL6R* expression [36, 37]. However, this seems to be cell type specific as IL-1β decreases *IL6R* expression in monocytes, but increases its levels in hepatocytes [38]. Therefore, it is possible that TGF-β effects on IL-6R expression could be specific for cartilage and TGF-β differently affects the IL-6 signaling pathway in other tissues or cell types. Interestingly, multiple studies report that *IL6R* expression is directly regulated by various miRNA's (miR-34a, miR-218, miR-590-5p) [39-41].

Several of these miRNA's have been linked to the TGF-β signaling pathway, and direct regulation of miR-34a by TGF-β has been described [40]. Follow-up studies are needed to show that TGF-β suppression of IL-6R expression is caused by TGF-β-induced miRNAs.

Besides regulation of IL-6R expression, it is possible that TGF-β blocks other mediators of IL-6 signaling downstream of the receptor as well. Interaction at the level of downstream mediators Smad3 and STAT3 has been extensively reported [42, 43]. These studies demonstrate that complex formation between Smad3 and STAT3 can lead to either inhibition of TGF-β signaling or synergy with STAT3 signaling cytokines, dependent on cell type or context [44]. Complex formation between Smad3 and STAT3 has not been studied in chondrocytes until now, but it is possible that these complexes are formed within our experimental setting and contribute to regulation of IL-6 signaling by TGF-β. However, we showed that TGF-β-mediated down regulation of IL-6R expression was the rate limiting factor, as rescuing IL-6R levels with soluble IL-6R abolished the inhibitory effect of TGF-β on IL-6 signaling.

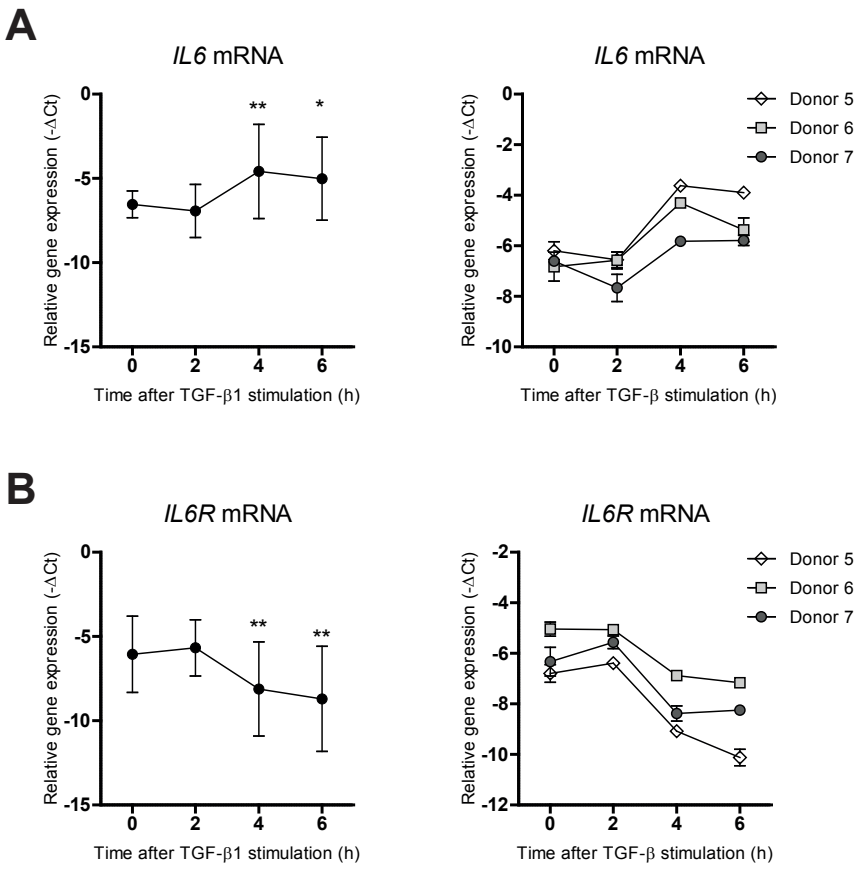
Next to a human chondrocyte cell line, we used human primary chondrocytes derived from macroscopically intact cartilage of OA patients in this study to investigate if our hypothesis held true in freshly isolated chondrocytes from different donors. Between the different individuals we observed diversity in the magnitude of TGF-β effect size, more specifically in the regulation of *IL-6* and *IL-6R* mRNA expression by TGF-β1. Moreover, we observed rapid p-STAT3 activation (5-15 min) in two donors. Direct activation of STAT3 was previously detected in hepatic stellate cells, but not in normal hepatocytes, indicating that this pathway is strongly cell-context dependent [45]. In this study, early p-STAT3 activation was caused by direct binding of JAK3 to TGF-βRI. It is therefore possible that the basal levels of TGF-β receptors (ALK5 vs ALK1), determines the early p-STAT3 peak in our setting [12]. However, this can also be caused by different factors, such as gender, age, disease severity or OA phenotype, which we were unable to study due to the use of anonymized material. It would be interesting for future research to establish whether rapid STAT3 activation represents a specific patient group.

Next to membrane-bound IL-6R present on the cell membrane, also a soluble form of the IL-6R exists. Soluble IL-6R results from shedding of membrane-bound IL-6R or from differential splicing of IL-6R mRNA and can form a complex with IL-6 and IL-6 receptor β (gp130), bypassing the need of membrane-bound IL-6R [46, 47]. Interestingly, endogenous soluble IL-6R can be detected in synovial fluid of OA patients and contributes to enhanced activation of chondrocytes by IL-6 [48-50]. This process, called trans-signaling, has been shown to negatively affect cartilage. For example, the IL-6/IL-6R complex can decrease transcription of the matrix components aggrecan and collagen type II in chondrocytes [34, 35]. This implies that in OA, the presence of soluble IL-6R may bypass the need of membrane bound IL-6R, which would abolish the protective effects of TGF-β on membrane bound IL-6R levels similar to our experiments where we add exogenous

rhIL-6R (Fig. 5). We propose that the presence of soluble IL-6R bypasses protective TGF-β effects, rendering cartilage sensitive again for catabolic IL-6 trans-signaling. These implications would highlight soluble IL-6R as an important target for therapies directed against IL-6.

In conclusion, we show that TGF-β1 dampens IL-6 signaling in chondrocytes, despite upregulating IL-6 levels. Moreover, we demonstrate that TGF-β1 inhibits IL-6 effects via down regulation of the IL-6R. This reveals a novel, protective effect of TGF-β potentially contributing to cartilage homeostasis. We suggest that when this protective effect is lost, due to the presence of soluble IL-6R during inflammation, cartilage is more sensitive to catabolic IL-6 signaling. This sheds light on the protective role of TGF-β in cartilage homeostasis and OA development.

SUPPLEMENTARY DATA



Supplementary Figure 1. Physiological levels of rhTGF-β1 (0.1 ng/ml) affect IL-6 and IL-6R expression in human articular chondrocytes. To investigate the effect of physiological levels of TGF-β1 on expression of IL-6 **(A)** and IL6R **(B)**, primary human chondrocytes of three donors were stimulated in triplicate with 0.1 ng/ml of rhTGF-β1 for 2, 4 or 6 hours. The mean of three donors is shown with corresponding 95% CI, and individual donors are plotted showing mean ± SD of technical replicates.

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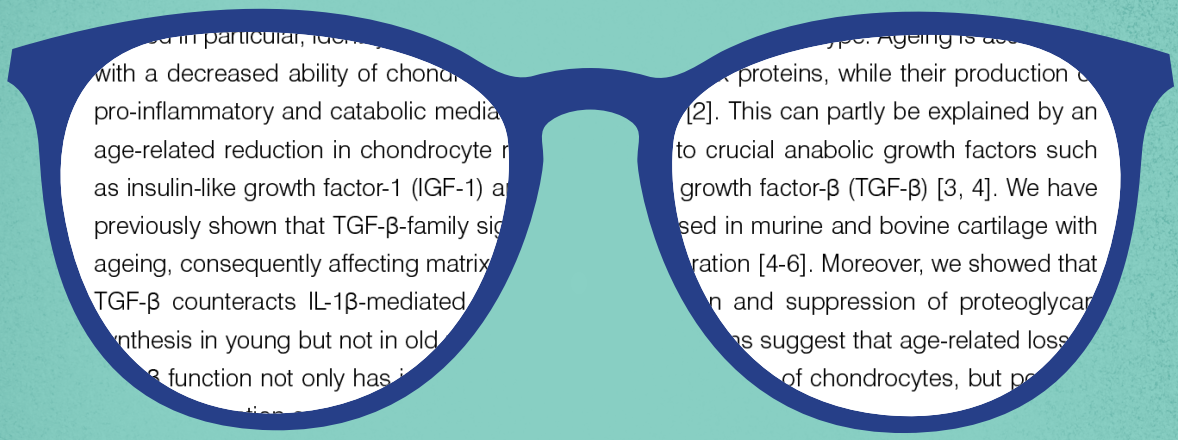

Chapter 5

Increased IL-6 receptor expression and signaling in ageing cartilage can be explained by loss of TGF-β-mediated IL-6 receptor suppression

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ABSTRACT

Objective

Osteoarthritis (OA) development is strongly associated with ageing, possibly due to age-related changes in transforming growth factor-β (TGF-β) signaling in cartilage. Recently, we showed that TGF-β suppresses interleukin (IL)-6 receptor (IL-6R) expression in chondrocytes. As IL-6 is involved in cartilage degeneration, we hypothesized that age-related loss of TGF-β signaling results in increased IL-6R expression and signaling in ageing cartilage.

Design

Bovine articular cartilage was collected and immediately processed to study age-related changes in IL-6R expression using qPCR and IHC (age-range: 0.5-14 years). Moreover, cartilage from young and aged cows was stimulated with rhIL-6 and/or rhTGF-β1 to measure IL-6-induced p-STAT3 using Western blot. Expression of STAT3-responsive genes was analyzed using qPCR.

Results

Expression of IL-6 receptor (*bIL-6R)* significantly increased in cartilage upon ageing (slope: 0.32, 95%CI: 0.20-0.45), while expression of glycoprotein 130 (*bGP130)* was unaffected. Cartilage stimulation with IL-6 showed increased induction of p-STAT3 upon ageing (slope: 0.14, 95%CI: 0.08-0.20). Furthermore, IL-6-mediated induction of STAT3-responsive genes like *bSOCS3* and *bMMP*3 was increased in aged compared to young cartilage. Interestingly, the ability of TGF-β to suppress *bIL6R* expression in young cartilage was lost upon ageing (slope: 0.21, 95%CI: 0.13-0.30). Concurrently, an age-related loss in TGF-β-mediated suppression of IL-6-induced p-STAT3 and *bSOCS3* expression was observed.

Conclusions

Ageing results in enhanced IL-6R expression and subsequent IL-6-induced p-STAT3 signaling in articular cartilage. This is likely caused by age-related loss of protective TGF-β signaling, resulting in loss of TGF-β-mediated IL-6R suppression. Because of the detrimental role of IL-6 in cartilage, this mechanism may be involved in age-related OA development.

INTRODUCTION

Osteoarthritis (OA) development is strongly associated with ageing, but this relationship is incompletely understood. It has become apparent that age-related changes in the musculoskeletal system contribute to OA development, together with other factors such as joint injury, genetic alterations and obesity [1]. Age-related changes in articular cartilage have been studied in particular, identifying major changes in chondrocyte phenotype. Ageing is associated with a decreased ability of chondrocytes to produce matrix proteins, while their production of pro-inflammatory and catabolic mediators is increased [2]. This can partly be explained by an age-related reduction in chondrocyte responsiveness to crucial anabolic growth factors such as insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) [3, 4]. We have previously shown that TGF-β-family signaling is decreased in murine and bovine cartilage with ageing, consequently affecting matrix repair and degeneration [4-6]. Moreover, we showed that TGF-β counteracts IL-1β-mediated nitric oxide production and suppression of proteoglycan synthesis in young but not in old mice [7]. These observations suggest that age-related loss of TGF-β function not only has implications for anabolic properties of chondrocytes, but possibly also for protection against pro-inflammatory stimuli.

The pro-inflammatory cytokine interleukin-6 (IL-6) is involved in the pathophysiology of several age-related diseases such as osteoporosis and Alzheimer's disease [8, 9]. IL-6 binds to the IL-6 receptor α subunit (IL-6R), followed by complex formation with signal transducer glycoprotein 130 (GP130) [10]. Besides IL-6 signaling, GP130 is involved in signal transduction of several other IL-6 family cytokines such as oncostatin M (OSM), IL-11, IL-27 and leukemia inhibitory factor (LIF) [11]. After assembly of the IL-6 receptor complex, associated Janus kinases (JAKs) are activated intracellularly, which in turn recruit and activate signaling mediators such as signal transducers and activator of transcription (STAT)3 [12]. IL-6 has a pivotal role in OA pathophysiology and cartilage degeneration, mostly via the induction of pro-inflammatory mediators and extracellular matrix degrading enzymes. In articular chondrocytes, IL-6 induces expression of matrix metalloproteinases (e.g. MMP-3 and MMP-13), as well as disintegrin and metalloproteinase with thrombospondin motifs (e.g. ADAMTS-4 and ADAMTS-5) [13-15]. Moreover, blockade of either IL-6 or its receptor in experimental OA models significantly protects against cartilage degradation [13, 16]. Systemic levels of IL-6 increase with normal ageing [17, 18], and are associated with OA development [19-21]. However, it is unclear whether there is a causal relation between IL-6 and age-related OA development. We previously showed that TGF-β is a potent inhibitor of IL-6 effects in chondrocytes, via decreasing IL-6R expression [22]. This protective mechanism might be disturbed in ageing cartilage, due to age-related loss of TGF-β signaling. In this study, we investigated if TGF-β-mediated suppression of IL-6R is lost in aged cartilage, and whether this subsequently results in increased IL-6 signaling. This could be a potential mechanism via which age-related changes in chondrocyte signaling predispose cartilage to OA development.

MATERIALS AND METHODS

Sample collection and stimulation

Bovine articular cartilage explants were harvested from metacarpophalangeal (MCP) joints of 86 individual cows with different ages. These donors were divided along the different experiments (Fig. 1, Fig. 2, Fig. 3, Fig. 4, Supplemental Figures), where each cow was considered as an independent donor. For experiments in which a broad age-range was studied, cartilage from cows aged 6 months up to 14 years old was included. Moreover, two age groups were defined to study young versus old cartilage. To exclude the effects of skeletal immaturity before the age of 3 years, cows aged 3-5 years were defined as young. Cows of approximately 10 years old (9-12 years) were defined as aged, as it was previously shown that cartilage of 10-year old cows shows well-known characteristics of ageing [6]. Cow legs were obtained within 3 h *post mortem* from a local abattoir, after which joints were immediately opened in a sterile environment to expose cartilage surface. Joints were examined for macroscopical signs of OA, and only cows with an intact cartilage surface were included. One joint per cow was used in this study. Full thickness cartilage explants were harvested from four condyles of the MCP joint using a 3 mm biopsy punch (Kai Medical). Cartilage explants from each individual cow were pooled and randomized to exclude bias due to sampling location. Per sample, four randomized explants from the same cow were used. For experiments in which isolated bovine chondrocytes were used (Supplemental Fig. 2), cartilage was digested overnight using Collagenase B (1.5 mg/ml, Roche Diagnostics) in DMEM/F12 medium (Gibco). Cartilage explants were shortly rinsed in saline and either flash-frozen in liquid nitrogen and stored at -80°C until later use, or cultured overnight in a 24-well plate (Cellstar; Greiner Bio-one International) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12 (Gibco)) without serum, supplemented with 100 mg/l pyruvate (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin in standard culture conditions (5% CO_2 (v/v), 37 °C, 95% humidity). After overnight serum starvation, explants were stimulated with rhBMP-9 (R&D Systems), rhIL-6 (Biolegend) and/or rhTGF-β1 (Biolegend) for time periods and dosages as indicated in figure legends. To reflect physiological levels of TGF-β1 in bovine cartilage, 1 ng/ml of rhTGF-β1 was used [23]. For RNA and protein studies, two samples (biological replicates) were included per experimental condition, each consisting of four explants from the same cow. For inhibitor studies, cartilage explants were pre-incubated with the ALK5 kinase inhibitor SB-505124 (5.0 μM, Sigma Aldrich) or TAK1 kinase inhibitor (5Z)-7-Oxozeaenol (0.5 μM, Tocris Bioscience) for 1 h before stimulation with rhTGF-β1. DMSO was used as vehicle control.

RNA isolation and quantitative real-time PCR

Cartilage explants were homogenized using a Mikro-dismembrator (1 min, 1500 rpm, Braun Biotech International). Total RNA was subsequently isolated with a RNeasy Fibrous Tissue Mini Kit (Qiagen) according to manufacturer's protocol. Thereafter, 8 μl sample was treated

with 1 µl of DNAse (Life Technologies) for 15 min at room temperature (RT) to remove possible genomic DNA, followed by inactivation of DNAse by incubation at 65°C with 1 µl 25 mM EDTA (Life Technologies). RNA was reverse transcribed into cDNA with single step RT reaction as previously described [22]. Gene expression was measured using SYBR green mastermix (Applied Biosystems) and validated primers (Biolegio; Table 1) using a StepOnePlus real-time PCR system (Applied Biosystems). The following amplification protocol was used: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were performed to confirm gene specific amplification. Data was expressed as relative gene expression corrected for the average of two reference genes (bGAPDH, bRPS14) depicted as −ΔCt, or corrected for reference genes and unstimulated control depicted as ΔΔCt. To determine the fold change in gene expression, log base 2 of ΔΔCt was calculated ($2^{-\Delta\Delta CT}$).

Protein isolation and detection

Cartilage explants were homogenized using a Mikro-dismembrator (1 min, 1500 rpm, Braun Biotech International). Samples were dissolved in 800 µl ice cold radioimmunoprecipitation assay (RIPA) buffer, with added 1 mM Na_3VO_4 and protease inhibitor cocktail (Complete, Roche) and incubated for 1 h on a roller bench at 4 °C. Samples were centrifuged for 3 min at 13,500 × *g* at 4 °C, where after the pellet was discarded and supernatant was boiled for 10 min at 95 °C. Proteins were separated using a 10% reducing bis-acrylamide SDS-PAGE gel, and transferred to a 0.45 µm pore nitrocellulose membrane (Life Sciences) using wet transfer (Towbin buffer, 2 h, 275 mA at 4 °C). Non-specific protein binding was blocked for 1 h with 5% bovine albumin serum to detect phosphorylated-STAT3 (p-STAT3) or with 5% non-fat dry milk (Campina) to detect GAPDH, dissolved in TBS-T (15 mM Tris-HCl, pH 7.4, 0.1% Tween). Membranes were incubated overnight at 4 °C with primary antibodies directed against p-STAT3 (polyclonal rabbit antibody, #9131, 1:1000, Cell Signaling) or GAPDH (mouse monoclonal antibody, 1G5, 1:10,000 Sigma Aldrich). After overnight incubation, membranes were incubated for 1 h with polyclonal Goat anti-Rabbit or Rabbit anti-Mouse coupled to horseradish peroxidase (1:1500, Dako) at RT. Enhanced chemiluminescence (ECL) was used to visualize proteins with ECL prime kit (GE Healthcare) and ImageQuant LAS4000 (Leica). Finally, blots were quantified using ImageJ.

Immunohistochemical analysis

For histological analysis, four explants per individual cow were harvested (5 young and 5 aged cows). Directly after isolation cartilage explants were fixed in 4% phosphate-buffered formalin (pH 7.0) for 16 h. Subsequently, samples were dehydrated using an automated tissue-processing apparatus (Thermo Scientific Excelsior AS) and embedded in paraffin. Sections of 4 µm were cut and mounted on Superfrost plus glass slides (Thermo Scientific). Sections were deparaffinized and washed with PBS. Endogenous peroxidase activity was blocked using 1% hydrogen peroxidase in methanol for 30 min at RT. Antigen-retrieval was performed using citrate buffer (0.1 M sodium citrate, 0.1M citric acid, pH 6.0) for 30 min at 37 °C. Hereafter, sections were blocked

Table 1. Template and sequence of the primers used in this study.

Name	Gene Symbol	Forward Sequence (5' -> 3')	Reverse Sequence (5' -> 3')
Glyceraldehyde 3-phosphate dehydrogenase	*bGAPDH*	CACCCACGGCA	TCTCGCTCCTGGAAGATGGT
Ribosomal Protein S14	*bRPS14*	CATCACTGCCCTCCACATCA	TTCCAATCCGCCCAATCTTCA
Interleukin-6 receptor	*bIL6R*	CGGCATCATCCTGAGGTTCAA	AGCTGGCCCAAAGAATACGA
Glycoprotein 130	*bGP130*	CCCACCTCATGCACTGTTGA	GTGGTGGATTGAGCTTCACTTT
Suppressor of cytokine signaling 3	*bSOCS3*	TCGGACCAGCGCCACTT	CACTGGATGCGCAGGTTCT
Cyclooxygenase-2	*bPTGS2*	GCACAAATCTGATGTTTGCATTC	GCACAAATCTGATGTTTGCATTC
Matrix metalloproteinase-3	*bMMP3*	AAACTCACCTCACGTACAGAATTG	TCCCAGACCGTCAGAGCTTT
Vascular endothelial growth factor A	*bVEGFA*	TGCTGCTCTACCTTCACCAT	ACATCCATGAACTTCACCACTTC
Tissue inhibitor of metalloproteinase-1	*bTIMP1*	GTTGTGAGGAATGCACAGTGTT	GTCCGTCCACAAGCAGTGA
Plasminogen activator inhibitor type 1	*bSERPINE1*	CGAGCCAGGCGGACTTC	TGCGACACGTACAGAAACTCTTGA

in 3% FCS/ 3% normal goat serum in PBS for 1 h at RT. Sections were incubated overnight at 4 °C with primary antibody directed against IL-6R in blocking solution (Abcam, ab128008, 0.2 µg/ml). A rabbit IgG isotype antibody (0.2 µg/ml, Dako) was used as a negative control. After staining with primary antibodies, a biotin-streptavidin detection system was used according to the manufacturer's protocol (Vector Laboratories). Antibody complexes were visualized with diaminobenzidine and counterstained with hematoxylin for 30 sec to visualize total cells. The number of positive cells was determined in a given threshold using ImageJ analysis, and expressed relative to total cell number. Per individual cow, four cartilage explants were stained for IL-6R protein, and pictures (100 x magnification) were taken from each individual explant for image analysis In each explant, the number of positive cells and total cells was counted using the cell counter plugin of ImageJ. For statistical analysis, the mean value per individual cow was used.

Statistical analysis

Quantitative data of individual cows of age range 0.5-14 years is plotted with a solid line representing the best fit regression analysis and 95% confidence interval (CI) as a dotted line (Fig. 1A, Fig. 2B, Fig. 4A, B). Quantitative data of young versus aged cows were expressed as a grouped column scatter of multiple biological replicates with displayed mean ± 95% CI. Before each analysis, Gaussian distribution was tested using the D'Agostino-Pearson Omnibus K2 test. The difference in IL-6R protein level between young and aged cows was tested using an unpaired two-tailed t-test (Fig. 1C). Differences in expression of IL-6 responsive genes were analyzed by comparing IL-6-stimulated conditions versus medium control (repeated measurements ANOVA), and by comparing young versus aged cows (normal ANOVA) both followed by Bonferroni's post-test to take multiple comparisons into account (Fig. 2C, Fig. 3). The effect of TGF-β on IL-6-mediated induction of p-STAT3 and *bSOCS3* in young and aged cows was equally analyzed (Fig. 4D, E). $P < 0.05$ was considered significant. All analyses were performed using Graph Pad Prism version 5.03 (GraphPad Software).

RESULTS

Expression of IL-6 receptor increases with age in bovine articular cartilage

Previously, it was demonstrated that bovine cartilage ranging 9 months - 10 years old shows well-known characteristics of normal ageing, such as thinning of the cartilage surface, reduced chondrocyte numbers, and decreased expression of matrix components such as collagen type 2 and aggrecan [6, 24]. Therefore, we collected 30 bovine donors of a similar age-range to study age-related changes in IL-6 receptor expression. On gene expression, bovine *IL6R (bIL6R)* levels increased with advancing age as determined with regression analysis (slope: 0.32, 95%CI: 0.20 - 0.45) (Fig. 1A). In contrast, we found no evidence for age-related changes in expression

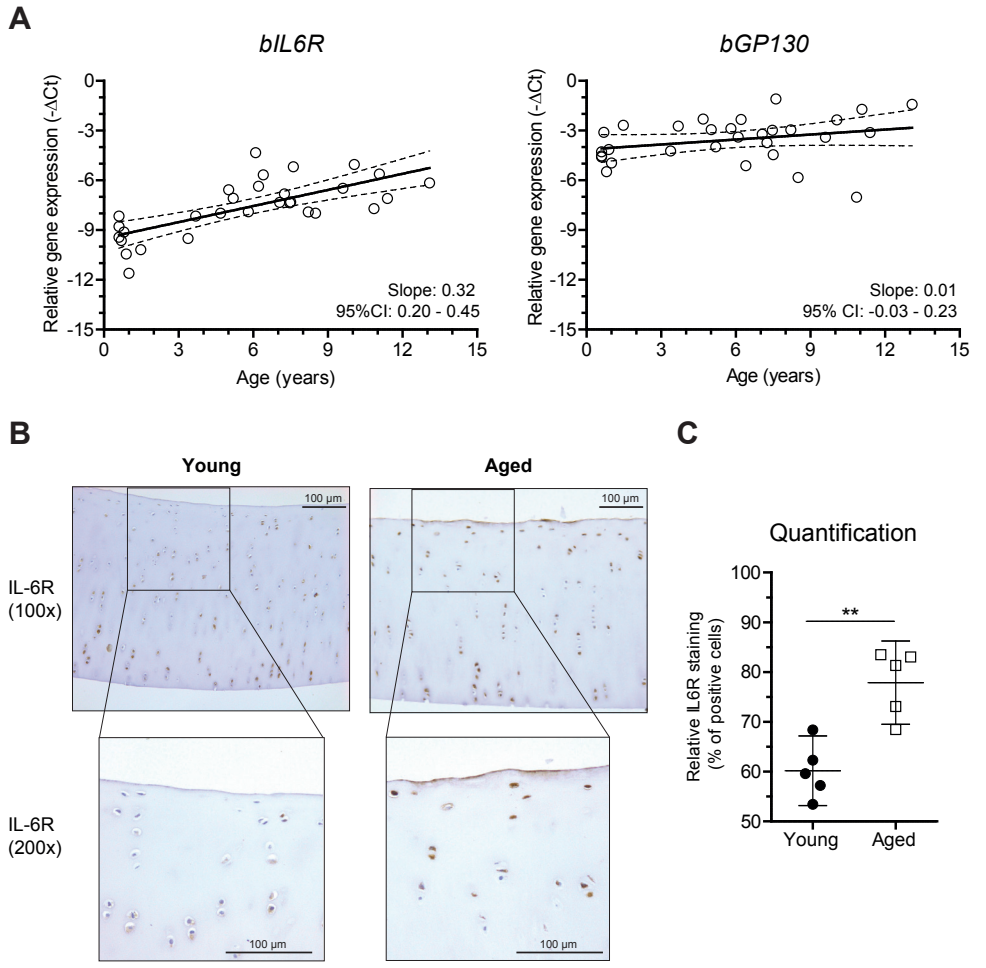


Figure 1. Age-related changes in interleukin-6 receptor expression in bovine cartilage. (A) Relative gene expression of bovine *interleukin-6 receptor* (*bIL-6R*) and *glycoprotein 130* (*bGP130*) as determined by qPCR in ageing bovine cartilage. Cartilage was collected from 30 different cows ranging from 6 months -14 years old and directly frozen in liquid N2 followed by RNA isolation and qPCR. Regression analysis (solid line) is depicted with corresponding confidence interval (dotted line). **(B)** Immunohistochemistry staining of IL-6R in young cartilage (3-5 years old) versus aged cartilage (9-12 years old). Cartilage was directly processed after collection. Pictures are representative of five cows per age group. Scale bars = 100 µm. **(C)** Quantification of percentage of cells positively stained for IL-6R in cartilage of young and aged cows. Using a fixed threshold in ImageJ, number of positive cells and total cells was counted in sections of four different cartilage explants per cow. Mean of four sections was calculated and plotted for five animals per age group. ** = $P < 0.01$ as measured by unpaired two-tailed t-test.

of *bGP130* (slope: 0.01, 95%CI: -0.03 - 0.23). To validate the age-related changes in *bIL6R* gene expression on protein level we performed immunohistochemical staining for IL-6R in young and aged cartilage. IL-6R was clearly detectable in bovine articular cartilage, with staining throughout the superficial, middle and deep zone of the cartilage (Fig. 1B). In contrast, no staining was observed when an isotype control antibody was used (Supplementary Fig. 1). In line with our observations on gene expression, the percentage of chondrocytes positively stained for IL-6R was increased in aged cartilage compared to young cartilage (17.70% increase, 95%CI: 8.65 - 26.76)(Fig. 1C).

Ageing increases IL-6 mediated phosphorylation of STAT3 and induction of *SOCS3* expression

Next, we determined if the observed age-related increase in IL-6R expression translated to enhanced activation of intracellular signaling. To study this, we stimulated cartilage explants *ex vivo* with rhIL-6 (100 ng/ml) for 30 min and measured activation of the main IL-6R downstream signaling protein STAT3 [12]. Stimulation with rhIL-6 resulted in a clear induction of p-STAT3 in bovine cartilage, which was markedly increased with advancing age (slope: 0.14, 95%CI: 0.08 - 0.20, n = 17) (Fig. 2A, B). In addition, we studied gene expression of the rapidly activated STAT3-target gene SOCS3 in young versus aged cartilage after stimulation with a high (100 ng/ml) or suboptimal (25 ng/ml) dose of rhIL-6. Dose of rhIL-6 was determined with a dose-response study in bovine chondrocytes (Supplemental Fig. 2). Expression of *bSOCS3* was increased by both concentrations of rhIL-6 compared to control medium, and reached maximum induction at 1 h after stimulation (Fig. 2C, D). Strikingly, induction of *bSOCS3* expression was higher in aged compared to young cartilage, but only in response to a suboptimal dose of rhIL-6 (2.3-fold, 1.2 ΔCt [95%CI: 0.52 - 1.91]). Stimulation with a high dose of rhIL-6 resulted in comparable induction of *bSOCS3* in young and aged cartilage (~4.9-fold, ~2.3 ΔCt [95%CI: 1.40 - 2.90]). Baseline expression values of *bSOCS3* and a more extensive time course analysis (1 h - 2 h - 4 h) are shown in (Fig. 2D), illustrating inter-individual variation in expression kinetics. Collectively, these data clearly show that IL-6-induced intracellular signaling via p-STAT3 and subsequent *SOCS3* gene expression are enhanced in aged cartilage.

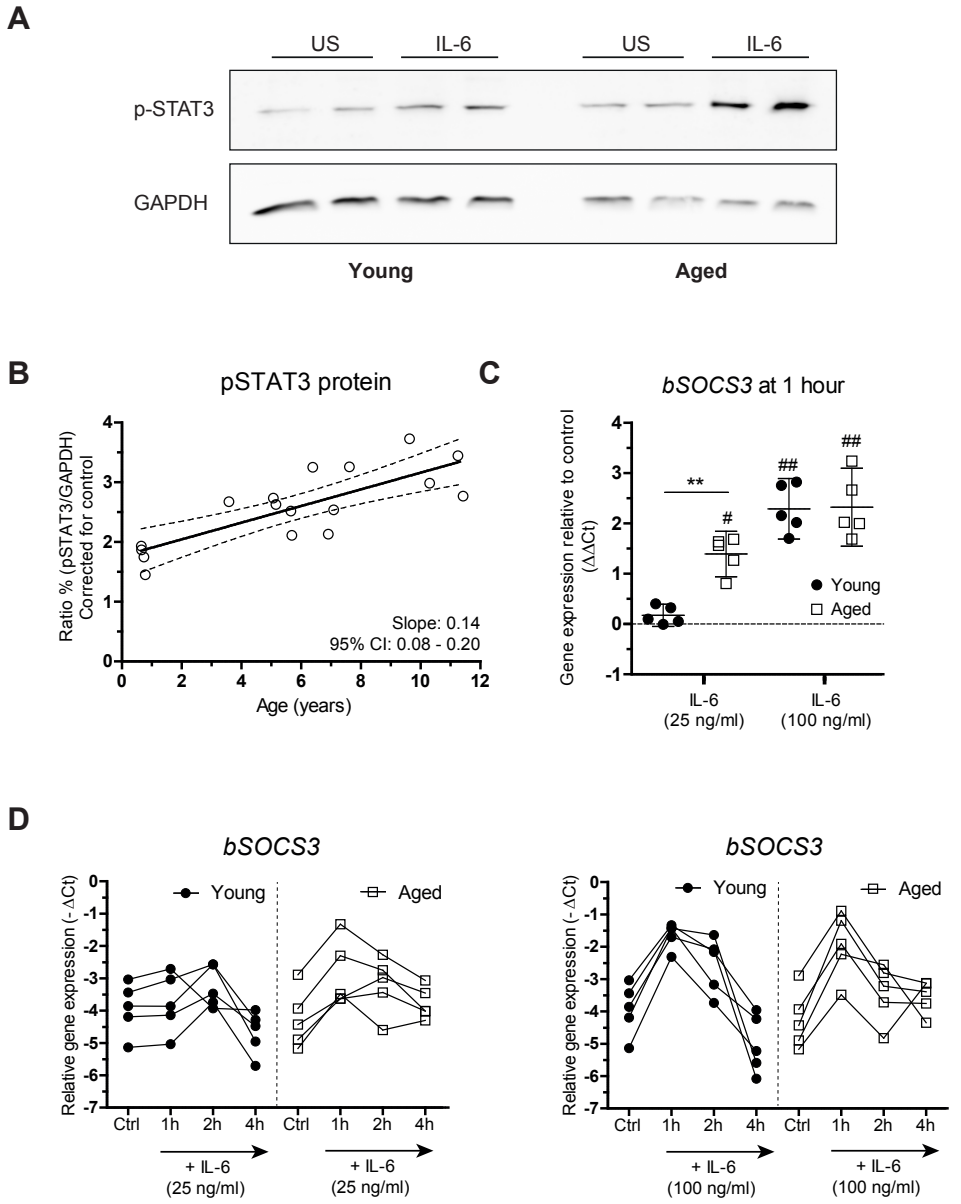


Figure 2. Aged cartilage shows increased induction of p-STAT3 and SOCS3 expression in response to IL-6. (A) To study age-related changes in IL-6 intracellular signaling in cartilage, cartilage explants from 17 different cows (6 months - 14 years) were stimulated *ex vivo* with rhIL-6 (100 ng/ml) for 30 min and STAT3 phosphorylation was visualized using Western blot. Representative image of a young cow (1 year) and old cow (10 years) are shown. Quantification of Western blot of all cows is shown in **(B)**. p-STAT3 levels were normalized to GAPDH levels and plotted as relative staining compared to control condition. Regression analysis (solid line) is depicted with corresponding confidence interval (dotted line). (**C, D)** To study age-

related changes in IL-6-mediated gene expression, cartilage from five young cows (3-5 years) and five old cows (9-12 years) was stimulated *ex vivo* with 25 ng/ml or 100 ng/ml of rhIL-6 for 1 h, 2 h, and 4 h. Expression of bovine *SOCS3* (*bSOCS3*) was measured using qPCR **(C)** Expression of *bSOCS3* after 1 h of IL-6 stimulation was corrected for reference genes (bGAPDH, bRPS14) and expressed relatively to unstimulated control (ΔΔCt). Plotted as grouped column scatter with mean ± 95% CI. **(D)** Relative expression of *bSOCS3* after 1 h, 2 h and 4 h corrected for reference genes (ΔCt). Plotted as symbols with connecting lines (each symbol represents mean of technical duplicate of individual cows). ** = $P < 0.01$ as measured by one-way ANOVA with Bonferroni's post-test. # = $P < 0.05$ compared to medium condition, ## = $P < 0.001$ compared to medium control tested with repeated measurements one-way ANOVA with Bonferroni's post-test. US: unstimulated

Aged cartilage shows higher expression of OA-related degenerative markers in response to IL-6

To determine the functional consequences of enhanced IL-6 receptor expression and signaling in cartilage upon ageing, we next analyzed age-related differences in IL-6 mediated activation of catabolic response genes related to cartilage degeneration. In aged cartilage, stimulation with rhIL-6 resulted in induction of catabolic mediators *bMMP3*, cyclo-oxygenase 2 (*bPTGS2*) and vascular endothelial growth factor-A (*bVEGFA)* compared to medium control after 4 h (*bPTGS2*) or 48 h (*bMMP3*, *bVEGFA*) (Fig 3A). Interestingly, we found no evidence for changes in expression of these three catabolic mediators in young cartilage in response to rhIL-6 stimulation. Comparison of young versus aged cartilage showed that *bMMP3* and *bVEGFA* were increased in aged cartilage in response to a high dose of rhIL-6 (100 ng/ml) (2.6-fold /1.4 ΔCt [95%CI: 0.66 – 2.07] and 1.9-fold / 0.94 ΔCt [95%CI: 0.36 – 1.52] respectively). Induction of *bPTGS2* was also higher in aged compared to young cartilage in response to a suboptimal dose of rhIL-6 (3.0-fold, 1.6 ΔCt [95%CI: 0.28 – 2.95]). In contrast, we could not find any evidence of age-related differences in IL-6-induced expression of the anti-catabolic mediator tissue inhibitor of metalloproteinases-1 (*bTIMP1*)(95%CI of difference: -0.01 – 1.17 [25 ng/ml], -0.75 – 0.42 [100 ng/ml]) after 48 h of stimulation. Supporting our results on gene expression, rhIL-6 stimulation also resulted in higher MMP3 protein expression in aged cartilage, but not in young cartilage (Fig. 3B). Taken together, this shows that age-related changes in IL-6 signaling result in increased expression of several OA-related degenerative factors in aged cartilage.

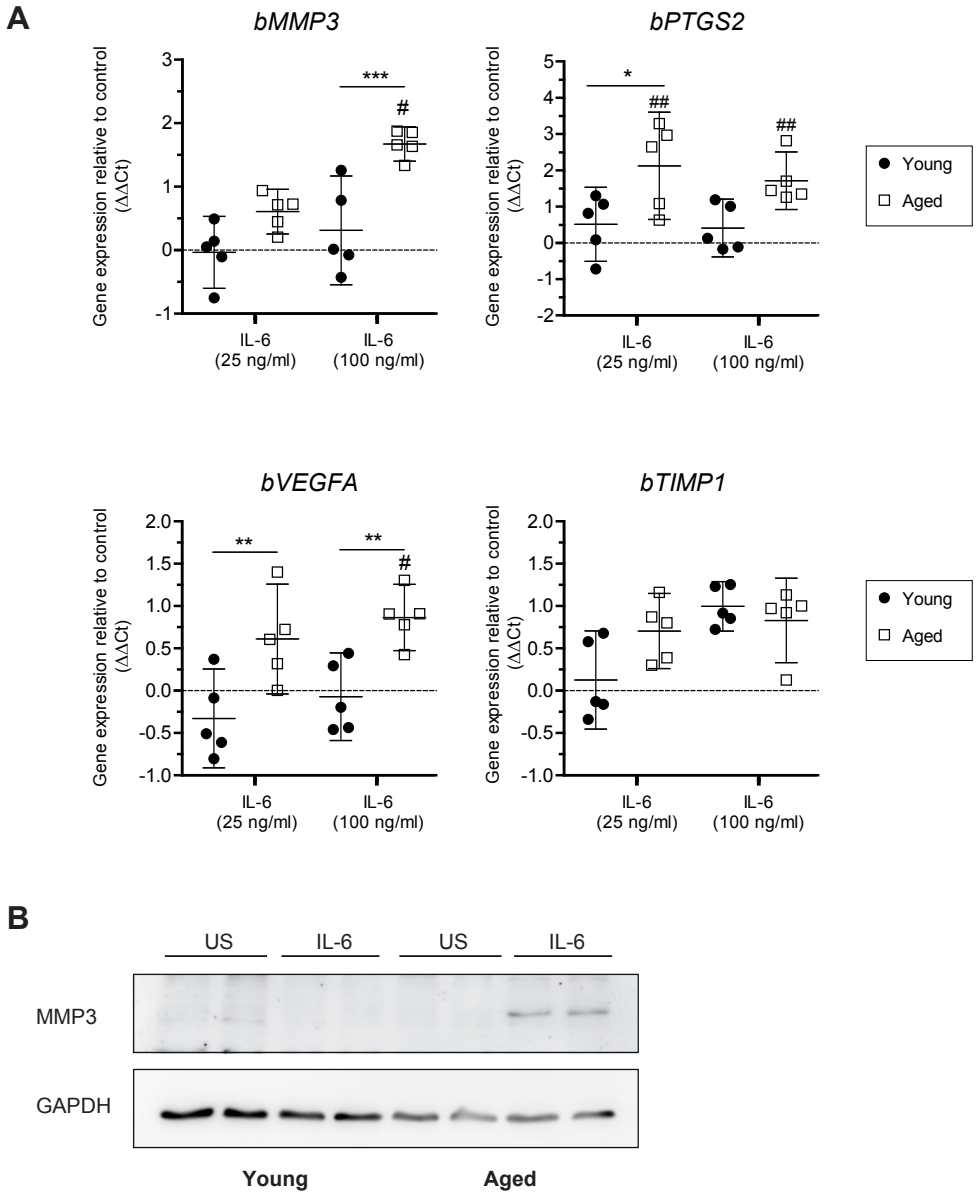


Figure 3. Ageing results in increased IL-6-induced expression of degenerative mediators in aged cartilage. (A) To study age-related changes in IL-6-mediated expression of markers related to cartilage degeneration, cartilage from five young cows (3-5 years) and five old cows (9-12 years) was stimulated ex vivo with 25 ng/ml or 100 ng/ml of rhIL-6 for 4 h (*bPTGS2*) and 48 h (*bMMP3, bTIMP1, bVEGF-A*). Expression values were corrected for reference genes (*bGAPDH, bRPS14*) and expressed relatively to unstimulated controls (ΔΔCt). Values were plotted as grouped column scatter with mean ± 95% CI. **(B)** MMP3 protein levels of cartilage explants stimulated with rhIL-6 (100 ng/ml, 24h). Western blot images are representative

of two young (both 5 years) and two old cows (9-10 years). * = P < 0.05; ** = P < 0.01, *** = P < 0.001 as measured by one-way ANOVA with Bonferroni's post-test. # = P < 0.05 compared to medium condition, ## = P < 0.001 compared to medium control tested with repeated measurements one-way ANOVA with Bonferroni's post-test.

Loss of TGF-β function with age results in increased IL-6 receptor expression and signaling

Previously, we demonstrated that TGF-β was able to inhibit IL-6 effects in articular chondrocytes by suppressing IL-6R levels [22]. Because TGF-β signaling decreases with advancing age in articular cartilage [4, 6], we hypothesized that this may be the underlying cause for the age-related increase in IL-6R levels. To study this, we stimulated cartilage explants *ex vivo* with rhTGF-β1 (1.0 ng/ml) for 24 h and analyzed IL-6R expression and signaling. To confirm that TGF-β signaling was indeed decreased with age in our data set, we first measured expression of *bSERPINE1*, a *Smad-3* dependent gene which was previously found to be affected in ageing cartilage [6, 25]. As expected, aged cartilage responded less to TGF-β than young cartilage, resulting in a negative regression coefficient (slope: -0.13, 95%CI: -0.02 to -0.23, n = 24) (Fig. 4A). To determine whether *bIL6R* expression was also no longer suppressed by TGF-β upon ageing, we depicted TGF-β-mediated suppression of *bIL6R* expression (Fig. 4B). Indeed, TGF-β suppression of *bIL6R* was reduced in bovine cartilage with advancing age (slope: 0.21, 95%CI: 0.13 – 0.30, n = 24). Previous studied showed that expression of the TGF-β receptor ALK5 decreases in articular cartilage with older age [5, 6]. Possibly, this age-related loss in ALK5 expression causes the reduced ability of TGF-β to suppress *bIL6R* expression and signaling in aged cartilage. To determine whether ALK5 is involved in TGF-β-mediated regulation of *bIL6R* expression, we made use of the ALK5 inhibitor SB-505124. As TGF-β only suppresses *bIL6R* expression in young cartilage (Fig. 4B), cartilage from cows aged 3-5 years was included in this experiment. Interestingly, TGF-β-mediated suppression of *bIL6R* expression was completely abolished by the addition of ALK5 inhibitor SB-505124 (Supplementary Fig. 3A). In contrast, we observed no effect of the TAK1 inhibitor (5Z)-7-Oxozeaenol, demonstrating that non canonical signaling via TAK1 is not involved. Furthermore, we observed no effect of stimulation with high-affinity ALK1 ligand BMP-9 on *bIL6R* expression, suggesting that ALK1 signaling does not modulate *bIL6R* expression (Supplementary Fig. 3B). On a functional level, pre-incubation with TGF-β (24 h, 1.0 ng/ml) profoundly suppressed IL-6-induced p-STAT3 in young cartilage (52% suppression, 1.32 mean difference [95%CI: 0.81 – 1.84), whereas this suppression was markedly reduced in aged cartilage (23% suppression, 0.76 mean difference [95%CI: 0.25 – 1.28) (Fig. 4C, D). Furthermore, pre-incubation with TGF-β suppressed IL-6-mediated induction of *bSOCS3* in young cartilage (2.5-fold decrease, 1.3 ΔCt [95%CI: 0.37 – 2.16), while we found no evidence for TGF-β-mediated suppression in aged cartilage (Fig. 4E).

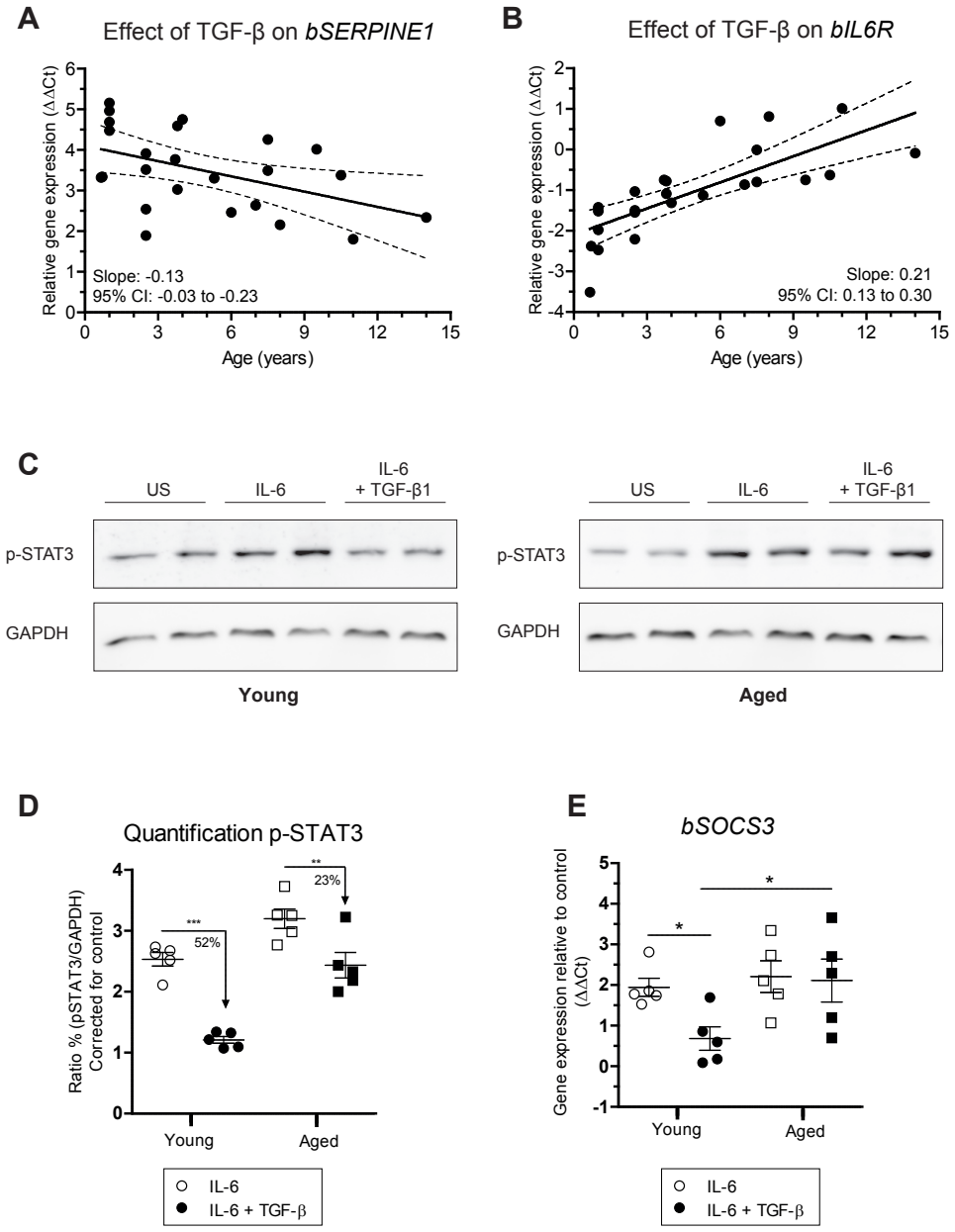


Figure 4. Age-related loss in TGF-β function to suppress IL-6 receptor expression and signaling. (A, B) To study TGF-β effects with ageing, cartilage explants of 24 cows (age-range: 6 months - 14 years) were stimulated ex vivo with TGF-β (1 ng/ml) for 24 h. Gene expression of *bIL6R* and *bSERPINE1* was analyzed using qPCR, corrected for reference gene expression and plotted relatively to unstimulated explants (ΔΔCt). Regression analysis (solid line) is depicted with corresponding confidence interval (dotted line). **(C, D, E)** To

determine the effect of TGF-β on IL-6 signaling in ageing cartilage, cartilage from five young cows (3-5 years) and five old cows (9-12 years) was pre-incubated with TGF-β (1 ng/ml) for 24 h, and hereafter stimulated with IL-6 (100 ng/ml). The IL-6 response was measured by induction of STAT3 phosphorylation after 30 min or induction of bSOCS3 expression after 1 h. **(C)** Representative image of a young cow (5 years) and old cow (11 years) are shown. **(D)** Quantification of Western blot of all cows is shown. p-STAT3 levels were normalized to GAPDH levels and plotted as relative staining compared to control condition. **(E)** Expression of *bSOCS3* was corrected for reference genes (*bGAPDH, bRPS14*) and expressed relatively to unstimulated control (ΔΔCt). Plotted as grouped column scatter with mean ± 95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by one-way ANOVA with Bonferroni's post-test. US: unstimulated

DISCUSSION

With ageing a number of changes accumulate in cartilage, including age-related alterations in chondrocyte signaling. In this study, we investigated age-related changes in IL-6 receptor expression and signaling in bovine cartilage as model of healthy cartilage. We report that IL-6R expression is markedly increased with age in articular bovine cartilage, resulting in enhanced IL-6-induced p-STAT3 signaling and expression of degenerative markers. Moreover, we showed mechanistic proof that age-related loss of TGF-β signaling may be the underlying cause for enhanced IL-6R expression and signaling in aged bovine cartilage.

The IL-6 signaling pathway is initiated by binding of IL-6 to the IL-6R, leading to complex formation with the signal transducing receptor GP130. Our study shows that expression of IL-6R increases in ageing bovine cartilage on both mRNA and protein level. In contrast, gene expression of the GP130 receptor, which also functions as a signal transducing receptor for other IL-6 family cytokines [11], is not changed in ageing bovine cartilage. This shows that ageing affects IL-6 signaling, but not necessarily GP130-mediated signaling via other IL-6 family cytokines.

Systemic IL-6 levels are increased with ageing and changes in IL-6 expression in ageing tissues have been extensively investigated [8, 26]. However, not many studies examined differences in IL-6R expression or signaling in relation to ageing. We are the first to show that IL-6-induced phosphorylation of STAT3 increased in articular cartilage upon ageing. In accordance with our observations, p-STAT3 levels were increased in human skeletal muscle biopsies of old compared to young donors (23-fold versus 5-fold respectively) 2 h after exercise [27]. Moreover, induction of STAT3 target genes (e.g. IL-6, SOCS3) was higher in old compared to young muscle tissue after exercise. However, whether IL-6 was the factor responsible for these effects, was not established in this study. Moreover, IL-6 can activate brain cells (microglia) in aged but not in adult mice, resulting in excessive production of pro-inflammatory cytokines [28]. Possibly, this is mediated via increased IL-6R levels in the ageing brain, similar to our observations in articular cartilage. However, this could also be mediated by different age-related changes such as increased levels

of IL-6 and/or soluble IL-6R in the cerebrospinal fluid. On the contrary, delayed activation of the IL-6 signaling pathway has been found in stem cells of older men [29]. This is potentially due to higher levels of the negative feedback inhibitor SOCS3 with older age (86% higher compared to young donors). We also found higher induction of *SOCS3* expression in response to IL-6 in aged cartilage, but this induction was not sustained over time and already decreased after 1 h. Moreover, we found no evidence that SOCS3 gene expression was affected by ageing at baseline level in articular cartilage.

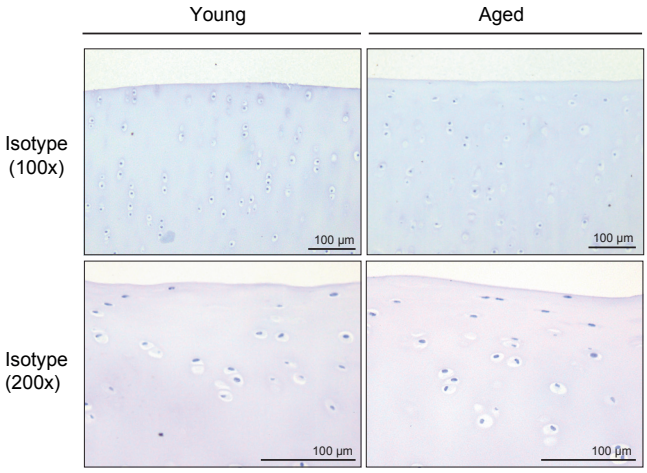
Surprisingly, male IL-6$^{-/-}$ mice developed more severe OA upon ageing [30]. This suggests that IL-6 may have protective role in age-related OA development in men, as opposed to our findings which suggest a detrimental role for IL-6 in ageing cartilage. These opposing findings can be explained in multiple ways. In a knock-out model, it is possible that different cytokines or growth factors compensate for the total loss of IL-6 which can bias the effect on cartilage damage and OA development. Moreover, complete, non-conditional IL-6 deficient mice were used in this study. As IL-6 is a pleiotropic cytokine with diverse functions throughout the body [31], this can in turn influence results of IL-6 in articular cartilage due to IL-6 deficiency from birth in e.g. bone or muscle tissue. Finally, in ageing mice, no markable inflammation was detected such as inflammatory infiltrate, exudate or synovitis, and systemic or local IL-6 levels were not measured [30]. During age-related OA development in humans, synovitis is commonly observed and levels of inflammatory mediators, amongst which IL-6, are systemically increased [8, 32, 33]. This raises the question whether murine ageing fully reflects human age-related OA development, and leaves open the possibility that IL-6 function differs in murine versus human ageing cartilage.

Due to the limited access to healthy human cartilage of different ages, we used bovine material as a model to study age-associated changes in healthy cartilage. Although we cannot exclude species-specific observations, ageing bovine cartilage displays many characteristics of ageing human cartilage [6, 34, 35]. Bovine cartilage can be obtained in a wide age range, and allows for examination of the cartilage surface before inclusion. Macroscopic selection of healthy cartilage allowed us to separate ageing from OA processes, which is difficult in murine or human cartilage where OA is often concomitant [6]. Interestingly, gene expression of catabolic markers *bMMP3*, *bPTGS2* and *bVEGFA* was increased in aged compared to young cartilage after IL-6 stimulation. In contrast, we found no evidence that IL-6 affected expression of anti-catabolic mediator TIMP-1, which has been reported previously as an IL-6-responsive gene in chondrocytes [36, 37]. Because of the detrimental role of MMP-3, COX-2 and VEGF-A in cartilage [13-15, 38, 39], our results suggest that enhanced IL-6 signaling in ageing cartilage may contribute to cartilage degeneration and OA development. Of note, we hardly found any regulation of *bVEGFA* expression in response to IL-6 in young cartilage, whereas this was observed in aged cartilage.

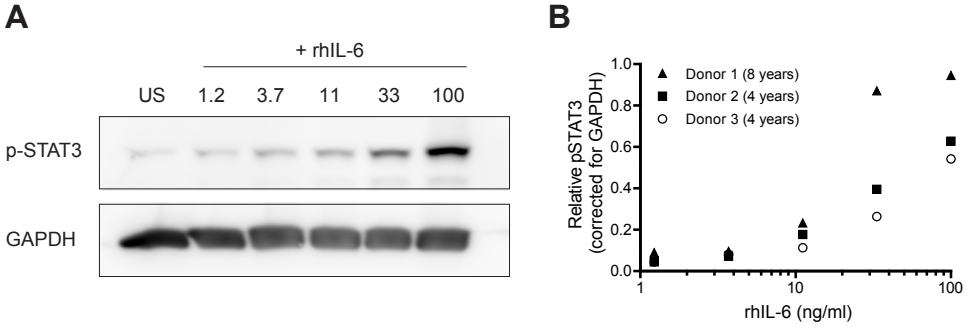
A plausible mediator causing the observed changes in IL-6R expression and signaling in aged cartilage could be TGF-β, a growth factor which is crucial for the maintenance of healthy articular cartilage [40]. Previously, we found that TGF-β potently suppresses IL-6R expression in chondrocytes and thereby inhibits IL-6 effects in chondrocytes [22]. In this study, we showed that TGF-β-mediated suppression of IL-6R expression is lost in aged cartilage. We previously observed decreased TGF-β signaling upon ageing in both murine and bovine cartilage. For instance, expression of TGF-β2 and -3 as well as corresponding TGF-β receptors, decreased strongly with age in murine cartilage [4]. Moreover, the number of p-Smad2 positive chondrocytes dropped markedly in aged murine cartilage, indicating a reduction in functional TGF-β signaling. Also in bovine cartilage, TGF-β-induced p-Smad3 signaling and expression of TGF-β receptors was reduced with age, which was most prominent for TGF-β receptor I (ALK5) [6]. This is in line with the finding that the ratio between ALK1 versus ALK5 increases with age in murine and human cartilage, potentially causing enhanced MMP-13 expression in OA [5]. Our results show that TGF-β-mediated IL-6R suppression is dependent on ALK5 kinase activity and is not mediated via ALK1 signaling. It is therefore likely that the reduced ability of TGF-β to suppress IL-6R in aged cartilage is due to a loss of ALK5 receptor expression with advancing age. It is difficult to directly proof that age-related loss of TGF-β function in cartilage causes enhanced IL-6R expression *in vivo*. However, our results clearly show that TGF-β loses its ability to suppress *bIL-6R* expression in aged cartilage. Moreover, we demonstrated that TGF-β has a reduced ability to suppress IL-6-mediated p-STAT3 signaling and *bSOCS3* expression in aged cartilage. Therefore, it is highly likely that age-related loss of TGF-β signaling mediates increased IL-6R expression and signaling in ageing cartilage.

In conclusion, we show that IL-6R expression is increased with advancing age in articular bovine cartilage, resulting in enhanced IL-6-induced p-STAT3 signaling and expression of degenerative mediators such as MMP-3. Moreover, we demonstrate that age-related loss of TGF-β function may be the underlying cause for increased IL-6R expression and signaling in aged cartilage. Due to the catabolic and pro-inflammatory role of IL-6 in OA development, we suggest that increased IL-6R levels with age predispose cartilage to degenerative changes, ultimately contributing to age-related OA development.

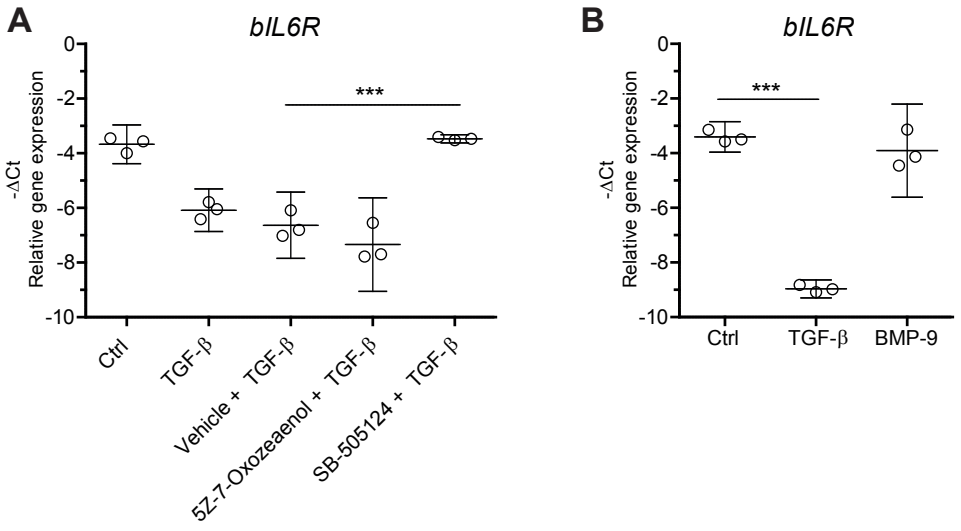
SUPPLEMENTARY DATA



Supplementary Figure 1. Isotype control staining of bovine cartilage. Cartilage from young (n = 5) and aged (n = 5) cows was directly processed after collection. Sections were stained with anti-IL-6R antibody (Fig. 1B) or IgG control (rabbit IgG). Representative pictures of isotype IgG negative control are shown per age group (100, 200x magnification). Scale bars = 100 µm.



Supplementary Figure 2. rhIL-6 dose-dependently increases p-STAT3 levels in bovine chondrocytes. To study the dose-response of bovine chondrocytes to rhIL-6 stimulation, bovine chondrocytes of three donors were stimulated with various concentrations of rhIL-6 (1.2, 2.7, 11, 33, 100 ng/ml) for 30 min. The IL-6 response was measured by induction of STAT3 phosphorylation as determined by Western blot. **(A)** Representative image of donor 2 is shown. **(B)** Quantification of p-STAT3 levels relative to GAPDH levels is shown for all cows. Age donor 1: 8 years, donor 2: 4 years, donor 3: 4 years.



Supplementary Figure 3. TGF-β-mediated suppression of bIL-6R expression is dependent on ALK5 kinase activity. (A) To study the role of TGF-β receptor ALK5 in TGF-β-mediated regulation of *bIL6R* expression, the small molecule inhibitor SB-505124 (5 μM) was used. Moreover, TAK1 dependency was studied using the (5Z)-7-Oxozeaenol (0.5 μM). DMSO was used as a vehicle control. Cartilage explants were pre-incubated with inhibitors for 1 h, and hereafter stimulated with rhTGF-β1 for a period of 24 h. **(B)** To study the effect of ALK1 signaling on *bIL6R* expression, cartilage explants were stimulated with high affinity ALK1 ligand rhBMP-9 (5 ng/ml) or rhTGF-β (1 ng/ml) for a period of 24 h. **(A, B)** As TGF-β only suppresses *bIL6R* expression in young cartilage, cartilage explants from young cows (3-5 years) were included (n = 3). Expression of *bIL6R* was analyzed using qPCR. Data is plotted as grouped column scatter with mean ± 95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by one-way ANOVA with Bonferroni's post-test. Ctrl: medium control.

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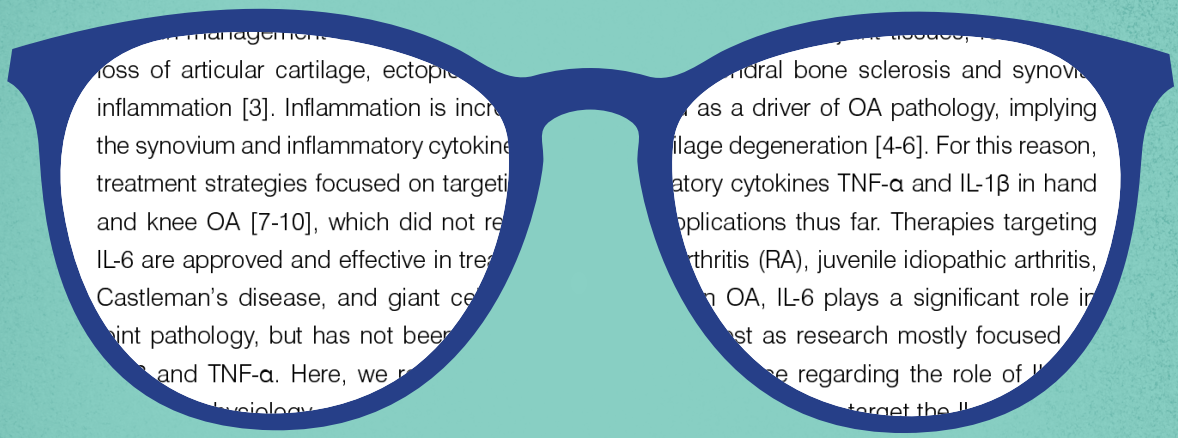

Chapter 5

A roadmap to target interleukin-6 in osteoarthritis

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ABSTRACT

Joint inflammation is present in the majority of osteoarthritis (OA) patients and pro-inflammatory mediators, such as interleukin-6 (IL-6), are actively involved in disease progression. Increased levels of IL-6 in serum or synovial fluid from OA patients correlate with disease incidence and severity, with IL-6 playing a pivotal role in the development of cartilage pathology e.g. via induction of matrix-degrading enzymes. However, IL-6 also increases expression of anti-catabolic factors, suggesting a protective role. Until now, this dual role of IL-6 is incompletely understood and may be caused by differential effects of IL-6 classic *vs* trans-signaling. Here, we review current evidence regarding the role of IL-6 classic- and trans-signaling in local joint pathology of cartilage, synovium and bone. Furthermore, we discuss targeting of IL-6 in experimental OA models and provide future perspective for OA treatment by evaluating currently available IL-6 targeting strategies.

Keywords

interleukin-6, osteoarthritis, , IL-6 trans-signaling, therapy, cartilage, synovitis

Key messages

- IL-6 signaling is actively involved in OA pathology, identifying IL-6 as a promising therapeutic target
- Differences between IL-6 classic *vs* trans-signaling explain the protective and degenerative IL-6 effects in joint tissues
- Specific targeting of IL-6 trans-signaling could be a superior treatment strategy in OA

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease with increasing incidence due to a rise in life expectancy and average body weight in western society [1, 2]. Currently therapies are focused on pain-management or eventually joint replacement. OA affects all joint tissues, resulting in loss of articular cartilage, ectopic bone formation, subchondral bone sclerosis and synovial inflammation [3]. Inflammation is increasingly accepted as a driver of OA pathology, implying the synovium and inflammatory cytokines in driving cartilage degeneration [4-6]. For this reason, treatment strategies focused on targeting pro-inflammatory cytokines TNF-α and IL-1β in hand and knee OA [7-10], which did not result in clinical applications thus far. Therapies targeting IL-6 are approved and effective in treating rheumatoid arthritis (RA), juvenile idiopathic arthritis, Castleman's disease, and giant cell arteritis [11]. Also in OA, IL-6 plays a significant role in joint pathology, but has not been a primary target of interest as research mostly focused on IL-1β and TNF-α. Here, we review the current state of evidence regarding the role of IL-6 in OA pathophysiology, and discuss potential therapeutic approaches to target the IL-6 signaling pathway in OA.

UNDERSTANDING THE COMPLEXITY OF THE IL-6 SIGNALING PATHWAY

Intracellular signaling cascades

IL-6 signaling starts by binding of IL-6 to the IL-6 receptor α subunit (IL-6R), followed by complex formation with a homodimer of glycoprotein 130 (gp130) [12]. The IL-6R has no signal transduction capacity and its expression is limited, e.g. to monocytes, hepatocytes, and certain leucocyte subsets [13]. In contrast, the signal-transducing receptor gp130 is ubiquitously expressed. Gp130 also functions as a β subunit for other IL-6 family cytokines, like oncostatin-M, IL-11, IL-27 and leukemia inhibitory factor [14]. After IL-6 receptor complex formation, the Janus kinases/signal transducers and activators of transcription (JAK/STAT) pathway is activated (Fig. 1), leading to recruitment and activation of STAT1, STAT3, and to a lesser extent STAT5 [15]. Besides canonical signaling via JAK/STAT, IL-6 activates non-canonical signaling via mitogen-activated protein kinase (MAPK) cascade (Ras-Raf-MEK-ERK pathway) and PI3K- protein kinase B (PkB)/Akt. IL-6-induced JAK/STAT is tightly controlled by negative feedback regulators, such as suppressor of cytokine signaling (SOCS) protein family and protein inhibitors of activated STATs (PIAS) [16, 17]. SOCS proteins are directly induced by gp130 cytokines, resulting in a negative feedback loop. SOCS3 has been identified as a specific inhibitor of IL-6 signaling and directly inhibits JAK-kinase activity [18, 19]. PIAS negative inhibitors are constitutively expressed and inhibit DNA-binding activity by binding to activated STAT-dimers.

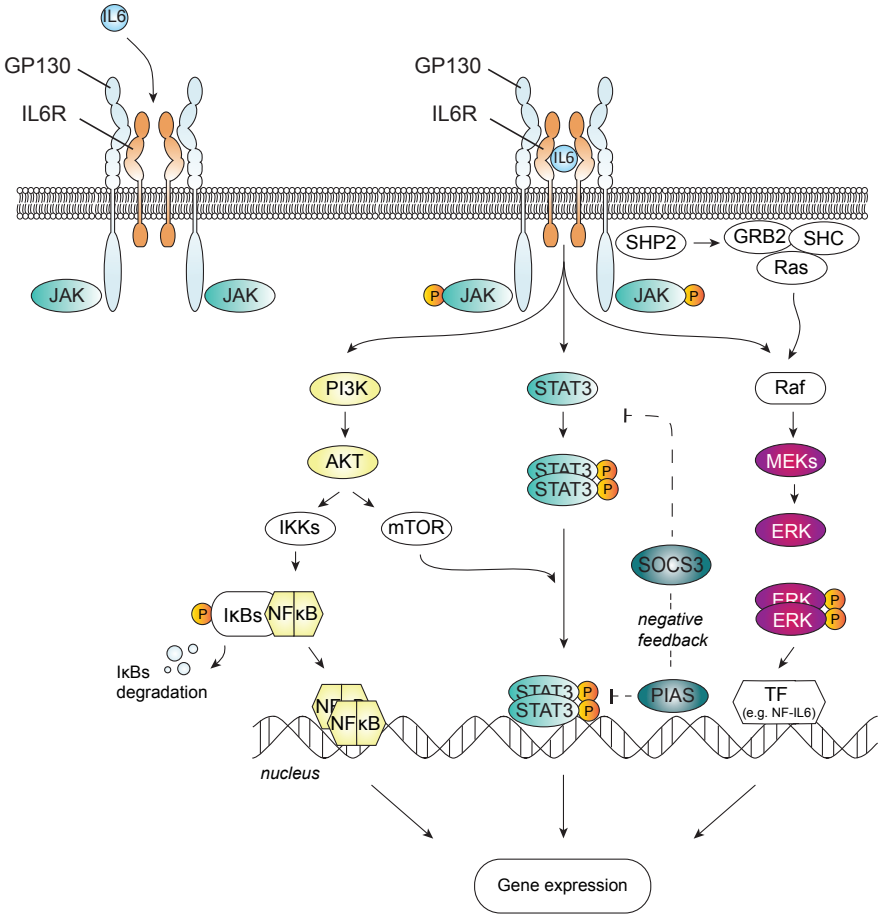


Figure 1. Overview of IL-6 signaling pathways. After IL-6 binding to the IL-6R, complex formation with gp130 initiates phosphorylation of JAKs resulting in activation of STAT3-, PI3K- and Ras-Raf-MEK-ERK signaling. Activated transcription factors (e.g. STAT3, NF-κβ and NF-IL-6) translocate to the nucleus to regulate target gene expression. SOCS and PIAS proteins negatively regulate IL-6-induced JAK-STAT signaling by blocking JAK-mediated activation of STAT3 (SOCS3), or by blocking DNA-binding activity of STAT3 (PIAS). Abbreviations: IL-6: interleukin-6; gp130: glycoprotein 130; JAK: janus kinase; STAT3: signal transducer and activator of transcription 3; PI3K: phosphoinositide 3-kinase; MAPK: mitogen-activated protein kinase; NF-κβ: nuclear factor kappa-light-chain-enhancer of activated B cells; NF-IL6: A nuclear factor for IL-6 expression; SOCS3: suppressor of cytokine signaling 3; PIAS: protein inhibitors of activated STATs.

Cytokine interplay and intracellular cross-talk

Interplay between IL-6 signaling pathways and other cytokines exists on multiple levels [14]. For example, other cytokines from the IL-6 family, like ciliary neurotrophic factor (CNTF) and IL-30, can also bind and activate the IL-6R, although with lower binding affinity compared with the CNTF- and IL-30 receptors [20, 21]. Furthermore, interplay between IL-6 and pro-inflammatory cytokine signaling, may restrict uncontrolled pro-inflammatory signaling [22]. For instance, IL-1β strongly inhibits IL-6-mediated acute phase reaction in the liver by directly inhibiting p38 MAPK-dependent STAT3 phosphorylation [22, 23]. More specifically, MAPK p38 and the transcription factor NF-κβ were identified as crucial regulators of the IL-6 signaling pathway [22]. Also, interplay between IL-6 and anti-inflammatory cytokines, such as TGF-β, is present at receptor level and at the level of intracellular mediators [24-26]. Crosstalk between STAT3 and Smad3, the main intracellular mediator of TGF-β signaling, exists in diverse pathophysiological conditions and leads to either synergistic or antagonistic actions depending on cell type and context [26].

Modes of IL-6 signaling

IL-6 has the unique ability to initiate signal transduction via different modes of receptor activation. Signaling via membrane-anchored IL-6R (mIL-6R) is termed classic signaling and is important for the acute-phase response, hematopoiesis, and central homeostatic processes [27](Fig. 2A). Interestingly, a soluble form of IL-6R (sIL-6R) can be produced by shedding of membrane-bound receptor or alternative splicing [28, 29]. sIL-6R can bind secreted IL-6, forming a complex that increases the half-life of IL-6 [30]. Signaling via sIL6R is called trans-signaling and greatly broadens the scope of IL-6 responsiveness, as any gp130-expressing cell can bind and respond to the IL-6/sIL-6R complex (Fig. 2B). IL-6 trans-signaling mainly regulates pro-inflammatory events and is implicated in numerous chronic diseases and cancers [27, 31]. Trans-signaling leads to stronger activation of IL-6 intracellular signaling routes, resulting in enhanced target gene expression, but how this works is still unclear [32, 33]. Possibly, restricted expression of mIL-6R limits activation of STAT3 via classic signaling, but not trans-signaling due to additional presence of sIL-6R [32]. Within our circulation, a soluble form of gp130 (sgp130) acts as a natural inhibitor of IL-6 trans-signaling by binding to the IL-6/sIL-6R with high affinity [31, 34-36]. Sgp130 therefore specifically inhibits IL-6 trans-signaling and does not affect classic signaling or recently discovered IL-6 cluster-signaling. IL-6 cluster-signaling involves membrane IL-6/IL-6R complexes on dendritic cells, which activate gp130 receptors on receiving T-cells resulting in the generation of pathogenic Th17-cells (Fig. 2C). [37]. Whether IL-6 cluster-signaling is also relevant in other biological settings remains to be investigated.

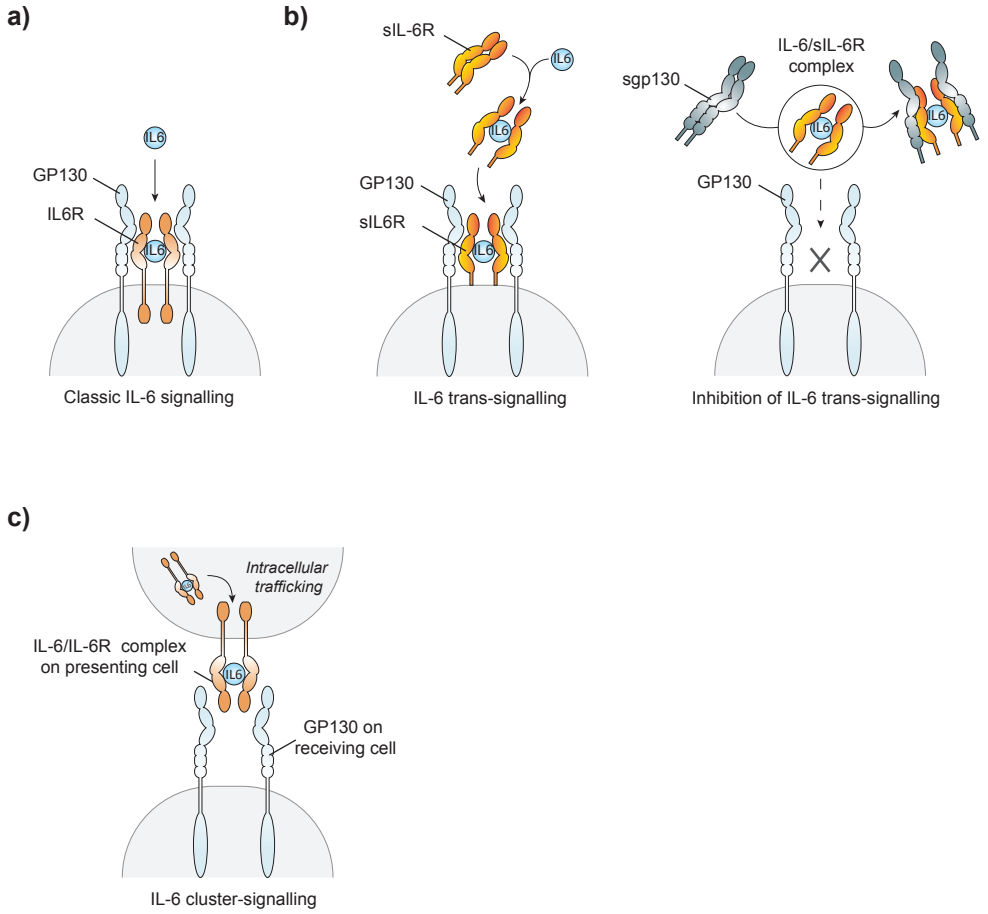


Figure 2. Modes of IL-6 signaling. **(a)** Classic IL-6 signaling involves cells expressing both membrane (m)IL-6R and gp130; free IL-6 binds to mIL-6R, forming a complex with gp130. **(b)** IL-6 trans-signaling is activated by pre-formed complexes of IL-6 and soluble IL-6R (IL-6/sIL-6R) and requires only gp130 expression on target cells. Soluble gp130 (sgp130) acts as a natural inhibitor of trans-signaling by specifically binding to IL-6/sIL-6R complexes. **(c)** IL-6 cluster signaling utilizes gp130 on receiving cells, activated by IL-6/mIL-6R complexes on presenting cells (e.g. dendritic cells). Abbreviations: IL-6: interleukin-6; IL-6R: IL-6 receptor; gp130: glycoprotein 130

LOCAL AND SYSTEMIC PERSPECTIVE: LEVELS OF IL-6 AND ITS SOLUBLE RECEPTORS IN OA

IL-6 levels relate to OA incidence and pathology

IL-6 was detected in OA synovial fluid (SF) as early as 1988. However, this did not result in follow-up studies, as IL-6 levels were lower compared with RA SF and healthy controls were not included [38, 39]. Later, it became clear that IL-6 levels were significantly increased in OA SF and serum compared with healthy individuals [40-42]. Furthermore, additional studies showed a clear relation between systemic IL-6 levels and OA incidence [43-45]. Increased circulating levels of IL-6 were predictive for knee OA and cartilage loss in 3 and 15 years in two independent follow-up studies [43, 44]. Moreover, a high innate capacity to produce IL-6, in response to lipopolysaccharide stimulation, was associated with hand OA development in 90-year old individuals [45]. Higher IL-6 levels in OA serum or SF also correlate with disease progression or severity of cartilage pathology [42, 46-48]. This suggests that IL-6 levels may reflect cartilage damage, which is supported by the fact that SF IL-6 levels are strongly increased in individuals with cartilage defects but no macroscopic signs of OA [49, 50]. When local *vs* systemic levels of IL-6 were compared in the same patients, IL-6 concentrations were higher in OA SF (119.8 ± 193.5 pg/ml) compared with plasma samples (3.1 ± 2.7 pg/ml) [51]. Furthermore, two patient subgroups can be identified based on IL-6 levels in OA SF, high producers (2022±526 pg/mL) *vs* average producers (132±19 pg/mL), of which high producers may particularly benefit from IL-6 targeted therapy [52].

Local production of IL-6 by joint tissues

It is now recognized that synovial inflammation is important in OA, and synovitis is observed in ~50% of OA patients [5, 53]. The synovium is an important producer of IL-6 in OA, for instance via (activated) synovial fibroblasts or plasma cells in the synovial lining [52, 54-56]. Besides the synovium, the infrapatellar fat pad (IFP) is an important source of IL-6. The IFP is the main fat tissue within the knee, and actively contributes to OA pathophysiology via production of pro-inflammatory mediators and adipokines [57]. Interestingly, the IFP from knee OA patients secreted significantly higher levels of IL-6, but not TNF-α and IL-1β, compared with abdominal fat tissue from the same patients [58]. Furthermore, IFP-conditioned medium cultured with traumatized cartilage explants caused IL-6-dependent glycosaminoglycan release [59]. Also, synovial fibroblasts from obese OA patients secreted higher levels of IL-6 compared with normal-weight patients [60], indicating that IL-6 may be particularly relevant in obesity-derived OA, especially as IL-6 plays a central role in cell metabolism [61].

Broadening our horizon: levels of soluble IL-6 receptors in OA

The ratio of IL-6 classic- *vs* trans-signaling is regulated by sIL-6 receptors [62]. Increased levels of sIL-6R in OA patients may direct future treatment towards specific inhibition of IL-6 trans-

signaling, while decreased sgp130 levels could indicate reduced negative feedback capacity. Unfortunately, studies investigating soluble IL-6 receptors in OA are scarce. Systemically, no differences were detected in sIL-6R levels in serum of healthy donors and OA patients [63], and as far as we know there is no study investigating systemic changes in sgp130 in OA. In OA SF both sIL-6R and sgp130 are present, but a comparison to healthy individuals is lacking [64-66]. Despite high levels of IL-6 production, it remains unclear if the synovium is a source of sIL-6 receptors in OA. In RA-derived material, cultured SF mononuclear cells produced sIL-6R, but not cultured chondrocytes or synovial cells [64]. However, this was not confirmed in OA. A potential source of sIL-6R in OA could be the IFP, which was shown to produce both IL-6 and sIL-6R [58], possibly resulting in IL-6 trans-signaling.

DIRECT EFFECTS OF IL-6 ON LOCAL JOINT TISSUES

Cartilage

As cartilage is the main OA affected tissue, previous studies mostly focused on identifying IL-6 effects in cartilage. However, IL-6 has both catabolic and protective effects in cartilage, which is still not completely understood. Early studies focused on IL-6 classic signaling and generally found protective effects of IL-6, such as production of tissue inhibitor of metalloproteinases (TIMPs) [67, 68]. Furthermore, IL-6 classic signaling slightly stimulated proteoglycan synthesis in human OA chondrocytes [49], and did not affect proteoglycan synthesis of human or bovine chondrocytes [69, 70]. However, detrimental effects of IL-6 classic signaling in cartilage have also been reported. IL-6 inhibited proteoglycan synthesis in human cartilage explants and rabbit chondrocytes [71-73]. Moreover, IL-6 suppressed collagen type II neo-synthesis and enhanced IL-1β-mediated proteoglycan degeneration in rabbit chondrocytes [73, 74]. Several studies show that IL-6 induces metalloproteinase (MMP)-3, MMP-13 and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) enzyme expression, which mediate cartilage degeneration [75-77]. Besides regulating matrix synthesis and degeneration, IL-6 induces matrix mineralization via formation of basic calcium phosphate crystals leading to proteoglycan loss [78]. Furthermore, IL-6 disturbs several other chondrocyte functions, resulting in decreased proliferation or increased oxidative stress generation [73, 79]. Of note, IL-6 directly induces SOCS3 expression, which can lead to insulin-like growth factor 1 desensitization in cartilage [80]. However, enhanced SOCS3 may also be protective as it restricts pro-inflammatory signaling in chondrocytes [81]. Altogether, this indicates that the definition of IL-6 classic signaling as only ‘protective’ is probably too simplified.

Generally, chondrocytes are considered to have low levels of mIL-6R which may limit STAT3 activation, but strong evidence for this is missing [32, 82]. In murine epiphysial chondrocytes, no mIL-6R expression was observed with flow cytometry [82]. In contrast, expression of mIL-6R

was observed in four donors of OA human chondrocytes on both mRNA and protein level [25]. Chondrocyte sensitivity for classic IL-6 signaling is determined by mIL-6R levels, which can be altered by hormones, cytokines and epigenetic factors [13]. The cytokine IL-1β increases mIL-6R expression in chondrocytes and hepatocytes [25, 83], which may explain synergistic effects of IL-1β and IL-6 in mediating cartilage degradation or collagen breakdown [71, 73, 84]. Cartilage injury induced by blunt trauma also potentiated IL-6-mediated expression of catabolic markers in bovine cartilage, but the underlying mechanism was not investigated [59, 85]. On the other hand, TGF-β decreases mIL-6R expression in chondrocytes, resulting in inhibition of classic IL-6 signaling [25]. Also mechanical compression of cartilage, which leads to active TGF-β signaling [86], inhibited catabolic effects of IL-6 and TNF-α combined [87]. Changes in mIL-6R expression in chondrocytes alter their sensitivity towards IL-6 classic signaling and may partly explain previous discrepancies regarding IL-6 effects in cartilage.

With respect to IL-6 trans-signaling in cartilage, functional studies also show contradictive results. Most studies conclude that IL-6 trans-signaling is detrimental for cartilage, as it inhibits proteoglycan synthesis and stimulates proteoglycan loss [72, 88]. Moreover, sIL-6R was required for full activation of JAK1/2 in bovine chondrocytes, resulting in decreased expression of matrix components and increased levels of cartilage degrading enzymes [89, 90]. On the other hand, soluble IL-6R also augmented production of anti-catabolic TIMPs in chondrocytes [67], which suggests that sIL-6R stimulates general IL-6 signaling and not only the catabolic response. In conclusion, there is substantial evidence for a direct role of IL-6 in regulating chondrocyte function and cartilage metabolism. While most studies report catabolic effects of IL-6 on cartilage, protective effects are also found. These discrepancies may be explained by functional differences in IL-6 classic *vs* trans-signaling, or disturbed expression of mIL-6R levels on chondrocytes.

Other joint tissues

As OA is a whole joint disease, direct effects of IL-6 on other joint tissues such as synovium, subchondral bone, and muscle tissue are also of interest [3]. Comparable to IL-6 effects in cartilage, mainly IL-6 trans-signaling was associated with detrimental effects in synovium. In OA- or RA-digested synovium, both IL-6 classic and trans-signaling increase production of TIMP, but only trans-signaling induces expression of ADAMTS4 [67, 68, 91, 92]. Moreover, IL-6 trans- but not classic signaling caused strong proliferation of RA synovial fibroblasts that could indicate a role in synovial hyperplasia or fibrosis [93]. However, in bone this distinction is less clear. On the one hand, IL-6 trans-signaling promotes osteoclast formation and consequently bone resorption, while classic signaling inhibits osteoclastogenesis [39, 94, 95]. On the other hand IL-6 trans-signaling has been shown to promote bone formation [96]. The dual effect of IL-6 trans-signaling on bone resorption and formation may be explained by variation in levels of the pro-osteoclastogenic factor receptor activator of nuclear factor kappa-B ligand (RANKL).

While high-levels of RANKL promote osteoclastogenesis, lower RANKL levels result in inhibition of osteoclast formation [97]. Finally, IL-6 may be directly involved in the process of OA-related muscle degeneration [98, 99]. Indeed, elevated levels of IL-6 are associated with reduced muscle endurance in elderly women with knee OA [100]. Moreover, increased levels of IL-6, STAT3 and SOCS3 have been detected in muscle tissue of knee OA patients [101]. However, IL-6 also mediates important anabolic processes in muscle tissue, such as muscle growth and myogenic differentiation [102]. This dual role of IL-6 in muscle tissue might be a result of functional differences in IL-6 classic- *vs* trans-signaling, but this is yet unknown. Thus, IL-6 classic and trans-signaling can affect joint tissues besides articular cartilage, but their respective functional effects and role in OA development remain elusive.

Evidence for a role of IL-6 in OA pathology: lessons learned from animal studies

The role of IL-6 in OA pathophysiology has been studied in several experimental OA models and mostly show a destructive role for IL-6 (Table 1). Induction of the destabilization of the medial meniscus (DMM) model in IL-$6^{-/-}$ mice resulted in marked reduction of cartilage destruction compared to wildtype mice [75]. Moreover, expression levels of MMP-3 and MMP-13 were significantly decreased in IL-$6^{-/-}$ mice compared to wildtype, indicating that IL-6 induces catabolic mediators in OA. This catabolic role of IL-6 was supported by overexpression of HIF-2α in wildtype *vs* IL-$6^{-/-}$ mice, causing OA-like cartilage destruction in wildtype but not IL-$6^{-/-}$ mice. Local injection of IL-6 into the knee joint, caused significantly increased cartilage degeneration and MMP-3 and -13 expression, revealing direct evidence for deleterious effects of IL-6 in OA [75]. Strikingly, despite marked evidence of IL-6 involvement in OA, only one study blocked IL-6 itself. Both systemic administration of a neutralizing antibody as well as anti-IL-6 siRNA resulted in decreased cartilage lesions and subchondral bone sclerosis in the anterior cruciate ligament transaction (ACLT) OA model [103]. Moreover, systemic treatment with an anti-IL-6-receptor neutralizing antibody (MR16) in the DMM model ameliorated the extent of cartilage pathology, synovial inflammation and osteophyte development [59]. This antibody is similar to Tocilizumab, which directly targets the human IL-6R and is clinically effective in several inflammatory diseases [11]. Moreover, blocking of the IL-6R using Tocilizumab resulted in cartilage preservation in a mouse model of ischemic osteonecrosis and significantly increased bone volume [104].

IL-6 is possibly mainly involved in trauma-related OA, as both the DMM and ACLT models reflect trauma-induced OA development. Also in humans, local IL-6 levels strongly increase upon cartilage trauma and associate with knee OA progression after previous meniscectomy [49, 50]. Systemic IL-6 levels also increase during natural ageing and are associated with several age-related diseases [105, 106]. However, there was no difference in cartilage degradation in wildtype or IL-$6^{-/-}$ mice after age-related OA development [107]. Male IL-$6^{-/-}$ mice even developed more cartilage damage, ectopic bone formation and subchondral bone sclerosis compared to male wildtype mice, while there was no difference in pathology of females. This suggests that

IL-6 has no pathological role in age-related murine OA, and even ameliorates OA pathology in male mice. Multiple studies show interplay between IL-6 and sex-hormones such as testosterone and estrogen [108-110], but it is unclear how sex hormones affect IL-6 function within the joint. Of note, no markable inflammation was detected in these mice including inflammatory infiltrate, exudate or synovitis [107]. This raises the question whether murine ageing fully reflects human age-related OA development, in which synovitis is commonly observed and levels of inflammatory mediators, amongst which IL-6, are systemically increased [5, 53, 105]. Besides age-related OA, there was no difference in OA pathology caused by collagenase-induced OA (CIA) between wildtype or IL-6$^{-/-}$ mice [107]. Although using conditional IL-6$^{-/-}$ mice, instead of constitutive knockouts, would more closely resemble physiological conditions, this suggests that other mediators may cause OA pathology in this model. However, to really exclude a role for IL-6 in CiOA and age-related OA, lack of IL-6 effects should be confirmed by independent studies. Functional differences in IL-6 classic *vs* trans-signaling may explain the contradictive results obtained in the different OA models. Unfortunately, all of the employed blocking strategies block both the classic- and trans-signaling pathway and current studies do not report sIL-6R levels. Specific inhibition of IL-6 trans-signaling in OA models might be extremely helpful to dissect detrimental *vs* protective effects of IL-6 in the future.

Blocking downstream of IL-6: targeting STAT3 in experimental OA

STAT3 is the most specific downstream mediator of the IL-6 signaling pathway, but is not solely activated by IL-6. Therefore, STAT3 activation in OA results from synergistic actions of several gp130 cytokines [14]. Hypothetically, targeting of STAT3 may be more successful compared with blocking IL-6, as other STAT3-activating cytokines also have catabolic and inflammatory effects on cartilage [112]. Indeed, repeated administration of a small molecule inhibitor against STAT3 (Stattic) in the DMM model resulted in stronger protection against cartilage degeneration and osteophyte formation compared with blocking IL-6R [59]. This additional effect may result from blockade of both IL-6 and OSM signaling via STAT3, based on the role of oncostatin M OSM in osteophyte proliferation and synovial inflammation [113, 114]. Inhibition of JAK2/STAT3 signaling in the ACLT model using the AG490 inhibitor, also led to considerable protection against cartilage degeneration and subchondral bone sclerosis [103]. However, mesoderm-specific deletion of STAT3 leads to expansion of growth plate hypertrophic chondrocytes and severe dysregulation of endochondral ossification, caused by STAT3-mediated activation of Sox9 in chondrocytes [115]. This phenotype is not observed in IL-6$^{-/-}$ animals [116], suggesting that other STAT3-activating cytokines may cause dysregulation of cartilage and bone development, such as LIF which is associated with reduced skeletal growth [117]. Recently, a novel gp130-small molecule modulator (RCGD 423) was discovered, which directed gp130 towards proliferative STAT3/c-Myc signaling, while inhibiting ERK/NF-κβ signaling. Therapeutic administration of the RCGD 423 compound, leading to STAT3 activation, resulted in reduced cartilage degeneration in a rat partial meniscectomy model [111]. This contradicts the earlier finding that STAT3 inhibition using

Table 1. Effect of targeting IL-6 signaling in experimental osteoarthritis models.

IL-6 targeting strategy	Animal (sex, strain, age)	OA model	Observations	Effect	References
General knock-out ($IL\text{-}6^{-/-}$)	Male C57BL/6 mice (10-12 weeks)	Surgically-induced post-traumatic OA (DMM)	↓ cartilage pathology	+	[75]
Systemic treatment with anti-IL-6 antibody	Male C57BL/6 mice (8 weeks)	Surgically-induced post-traumatic OA (ACLT)	↓ cartilage pathology	+	[103]
Systemic treatment with STAT3 inhibitor_(AG490)	Male C57BL/6 mice (8 weeks)	Surgically-induced post-traumatic OA (ACLT)	↓ cartilage pathology ↓ subchondral bone sclerosis	+	[103]
Systemic treatment with anti-IL-6R antibody (MR16-1)	Male C57BL/6 mice (10 weeks)	Surgically-induced post-traumatic OA (DMM)	↓ cartilage pathology ↓ osteophyte formation ↓ synovitis	+	[59]
Systemic treatment with STAT3 inhibitor (Stattic)	Male C57BL/6 mice (10 weeks)	Surgically-induced post-traumatic OA (DMM)	↓ cartilage pathology ↓ osteophyte formation	+	[59]
General knock-out ($IL\text{-}6^{-/-}$)	Male C57BL/6 mice (18-23 months)	Age-related OA (Spontaneous OA)	↑ cartilage loss ↑ subchondral bone sclerosis	-	[107]
General knock-out ($IL\text{-}6^{-/-}$)	Female C57BL/6 mice (18-23 months)	Age-related OA (Spontaneous OA)	No difference	No effect	[107]
General knock-out ($IL\text{-}6^{-/-}$)	Male and female C57BL/6 mice (3-4 months)	Chemically-induced joint instability OA (CiOA)	No difference	No effect	[107]
Intra-articular treatment with gp130 modulator (RCGD 423)	Male Sprague-Dawley rats (3-4 months)	Surgically-induced post-traumatic OA (Partial meniscectomy)	↓ cartilage pathology ↓ osteophyte formation ↓ chondrocyte proliferation	+	[111]

Abbreviations: IL-6: interleukin-6; DMM: destabilization of the medial meniscus; ACLT: anterior cruciate ligament transection; CiOA: collagenase-induced OA;

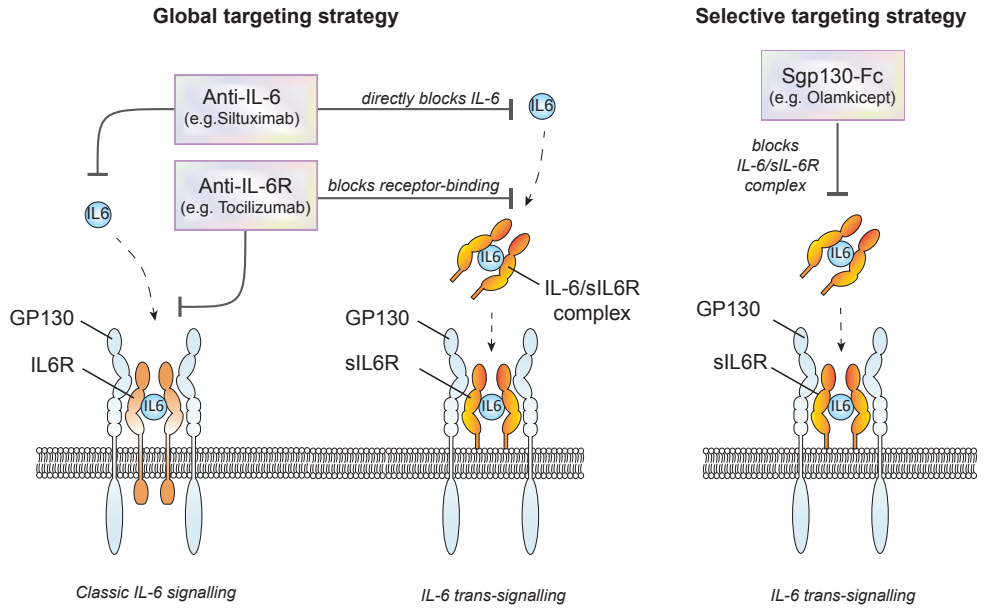
Stattic protects against cartilage degeneration [59]. It is possible that the proliferative effect of the RCGD423 inhibitor is caused by the strong activation of c-Myc, as LIF-driven c-Myc activation is critical for chondrocyte survival and proliferation in fetal cartilage [111]. These opposing results indicate that the ultimate result of STAT3 inhibition, beneficial or detrimental, is strongly context-dependent and determined by the integrated signal of multiple gp130 cytokines. While targeting of STAT3 may seem promising in experimental OA, this might prove difficult in OA patients due to large differences in severity and incidence of inflammation, and heterogeneity in STAT3-activating cytokines or growth factors [53]. This makes it difficult to predict the outcome of STAT3 inhibition in OA and argues for the simpler approach of directly targeting gp130 cytokines, such as IL-6, upstream of STAT3.

UNDER CONSTRUCTION: IL-6 TARGETED THERAPY IN OA

Currently multiple therapeutic strategies exist to effectively target the IL-6 signaling pathway, and are safely applied for the treatment of several inflammatory diseases. For example, the IL-6R targeting antibody tocilizumab has been approved for treatment of RA, juvenile idiopathic arthritis, Castleman's disease and recently also for giant cell arteritis [62, 118]. Currently, no therapies targeting IL-6 signaling are approved for treatment of OA. This may change in the near future, as tocilizumab is being tested in a phase 3 randomized controlled trial in patients with refractory hand OA (ClinicalTrials.gov NCT02477059).

Previous studies showed that levels of IL-6 in SF can vary between different joints, which could direct future IL-6 targeted therapy towards relevant patient subgroups. For example, levels of IL-6 were strikingly higher in knee OA SF compared with carpometacarpal joint fluid [119]. Moreover, more IL-6 was detected in post-traumatic wrist OA compared with knee OA patients [120]. As inflammation is strongly linked to structural damage in hand OA patients [121-123], this patient group may be very suitable to study the consequences of blocking pro-inflammatory cytokines, such as TNF-α [124] and IL-1β [7] and now IL-6. Besides stratification of patients based on joint-location, treatment choice could also be based on OA subtypes. Post-traumatic OA is a common form of OA, developing after joint injury (e.g. anterior cruciate ligament or meniscus injury) [125]. In these patients, there may be a therapeutic window after injury, in which targeting of inflammatory mediators may prevent the development of further damage. During joint injury, such as anterior cruciate ligament rupture, levels of IL-6 in SF are highly increased up to 1000-fold [126, 127], and a sudden increase in IL-6 levels has also been found after focal cartilage damage [49, 50]. This suggests that inhibition of IL-6 shortly after joint injury may be a promising treatment strategy to prevent the development of post-traumatic OA, however, the optimal therapeutic window to prevent further damage is still unknown.

Due to the success of tocilizumab, novel IL-6 pathway inhibitors have been developed, such as biologics targeting IL-6R (vobarilizumab, satralizumab, sarilumab), IL-6 (siltuximab, olokizumab, sirukumab, clazakizumab and MEDI 5117), IL-6 trans-signaling (olamkicept), or small molecule inhibitors directed against JAKs or STAT3 [128]. Multiple inhibitors have been developed and clinically tested that target JAK-kinases or STAT3 directly [11]. Although some of these compounds are clinically effective in RA, such as tofacitinib and baricitinib (pan-JAK inhibitors), they have not been tested in OA patients [129]. Yet, there are pre-clinical indications that JAK/STAT inhibition could be effective in OA. For instance, tofacitinib inhibited cytokine-induced proteoglycan loss and restored collagen type II synthesis in bovine cartilage explants [130]. In addition, animal studies indicate protective effects of JAK/STAT inhibition in experimental OA [59, 103]. However, targeting of JAK/STAT signaling also results in inhibition of multiple cytokines including IL-10, IL-4 and IGF-1, which have a beneficial role in joint biology and OA development [14, 131, 132]. As OA is a very heterogeneous disease with large differences in severity of inflammation and cytokine profile [53], it will be difficult to predict outcome of JAK/STAT inhibition in OA patients. Therefore, the simpler approach of targeting one cytokine, like IL-6, might be a safer strategy. As several therapeutics have been developed that target IL-6 signaling via a different mechanism, the comparison of these treatments will greatly enhance knowledge about the role of IL-6 in disease. Tocilizumab, for instance, blocks all IL-6 signaling pathways (classic and trans-signaling, and potentially also cluster-signaling [37], because it inhibits IL-6 binding to both mIL-6R and sIL-6R (Fig. 3)[133]. In contrast, olamkicept specifically targets the IL-6/sIL-6R complex, thereby only inhibiting IL-6 trans-signaling, and not classic signaling. Olamkicept is a fusion protein consisting of two soluble human gp130 proteins fused with the Fc region of human IgG (sgp130Fc) [128]. Accordingly, olamkicept blocks pro-inflammatory events of IL-6 trans-signaling, while simultaneously allowing homeostatic effects of IL-6 classic signaling. Olamkicept was already successfully used in treating experimental Crohn's disease and is now in clinical trials for inflammatory bowel disease and active ulcerative colitis [11, 134]. Specific inhibition of IL-6 trans-signaling could be a preferred future treatment strategy, as several side effects of tocilizumab have already been reported that may result from inhibition of IL-6 classic signaling, such as impairment of the acute phase response [128]. Olamkicept might also be a promising therapy for OA, as several studies demonstrated catabolic and pro-inflammatory effects of IL-6 trans-signaling in cartilage and synovial tissue [72, 88, 92, 93]. Thus, even if tocilizumab does not prove effective in hand OA, specific inhibition of IL-6 trans-signaling may hold additional promise. Nonetheless, additional pre-clinical research will first be needed as the relevance of IL-6 trans-signaling in experimental OA has not yet been demonstrated.



Agent	Target	IL-6 signalling interference
Anti-IL-6 (humanized monoclonal antibody)	IL-6	Classic and trans-signalling
Anti-IL-6R (humanized monoclonal antibody)	IL-6R	Classic and trans-signalling
Sgp130-Fc (fusion protein)	IL-6/IL-6R complex	Trans-signalling

Figure 3. Current IL-6 targeting strategies. Anti-IL-6 monoclonal antibodies (e.g. Siltuximab) directly target IL-6, thus blocking both classic and IL-6 trans-signaling. IL-6R targeting antibodies (e.g. Tocilizumab) block binding of IL-6 to the IL-6R (both mIL-6R as well as sIL-6R), thereby inhibiting IL-6 classic and trans-signaling pathways. The sgp130Fc fusion protein (e.g. Olamkicept) was developed to specifically target IL-6 trans-signaling, and only binds to the IL-6/sIL-6R complex. Sgp130Fc does not bind to membrane IL-6R or free IL-6, therefore allowing classic IL-6 signaling to continue. Abbreviations: IL-6: interleukin 6; mIL-6R: membrane IL-6 receptor; sIL-6R: soluble IL-6 receptor; sgp130: soluble glycoprotein 130.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this review, we focus on the unique ability of IL-6 to signal via the classic- and trans-signaling pathway, and discuss the opposing effects of these signaling routes in OA pathophysiology and treatment. Levels of IL-6 are increased in SF and serum of OA patients, and relate to disease incidence and pathology. In contrast, regulation of sIL-6R in OA, which controls activation of IL-6 trans-signaling, has been overlooked until now and warrants further research. In local joint tissues such as cartilage, synovium and bone, IL-6 classic signaling results mainly in protective effects, while trans-signaling leads to pro-inflammatory and catabolic effects. However, it is highly likely that local regulation of IL-6R levels also determines IL-6 outcome to a great extent. Current evidence of IL-6 blockade in experimental OA shows that therapeutic targeting of the IL-6 pathway could be a promising treatment strategy to reduce cartilage damage, synovial inflammation and subchondral bone pathology in OA patients. Moreover, we propose that specific blockade of IL-6 trans-signaling could be a superior treatment strategy, which may result in inhibition of deleterious IL-6 effects in OA, while maintaining protective IL-6 signaling via the classic pathway.

SEARCH STRATEGY

Articles were selected using the PubMed search engine. To select articles regarding IL-6 in OA, we used following search terms present in title or abstract [tiab]: “interleukin-6” in combination with search terms covering the topics in this review including “osteoarthritis”, “cartilage”, “synovium”, “bone”, “muscle”, “infrapatellar fat pad” and “therapy”. Relevant synonyms were included using MeSH terms. Title and abstract of articles were screened for relevant topics as listed in this review. Non-English articles, and articles in which IL-6 was only used as a marker of inflammation were excluded. In addition, reference lists of cited articles and articles in our personal databases were screened for eligibility. Search includes articles published up to January 2020.

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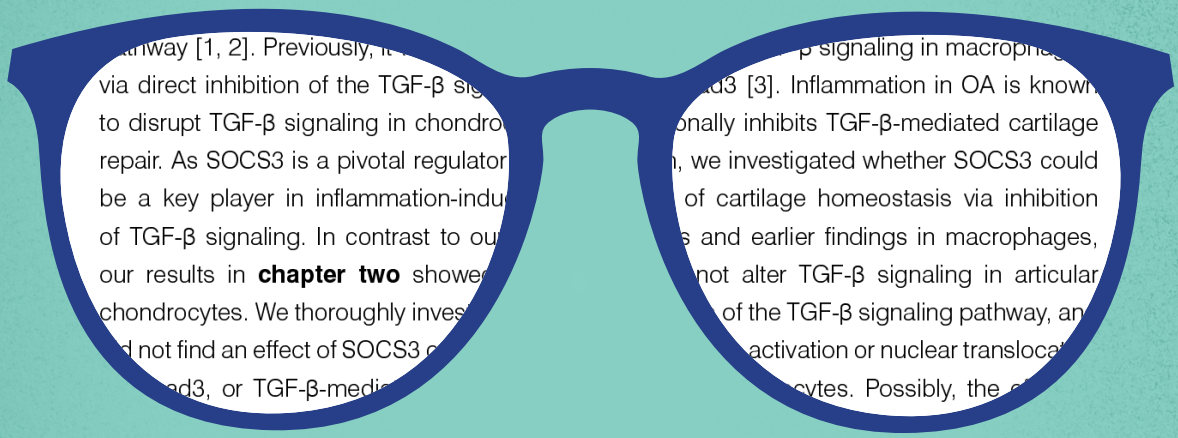

Chapter 6

Summary, general discussion and future perspectives

SUMMARY

Cartilage degeneration in OA is caused by disbalance between anabolic and catabolic processes, which for a large part is regulated by signaling proteins such as pro-inflammatory cytokines and growth factors. In healthy cartilage, the protective growth factor TGF-β maintains cartilage homeostasis and has important anti-hypertrophic and anti-inflammatory properties. However, pro-inflammatory cytokines can interfere with TGF-β signaling, contributing to disrupted cartilage homeostasis and eventually cartilage degeneration. Levels of the pro-inflammatory cytokine IL-6 are increased in OA, and directly contribute to cartilage degeneration e.g. via increasing expression of matrix degenerative enzymes. Yet, it remains to be elucidated whether IL-6 affects TGF-β signaling in chondrocytes, or vice versa. In this thesis, we set out to unravel the interplay between components of the IL-6 and TGF-β signaling pathway in articular cartilage and determine whether this could play a role in ageing and OA.

Inflammation-induced SOCS3 does not alter TGF-β signaling in chondrocytes but hampers cartilage formation in a three-dimensional MSC pellet culture model

First, we focused on interplay between TGF-β signaling and SOCS3, a protein that is rapidly induced by inflammation and functions as an important negative regulator of the IL-6 signaling pathway [1, 2]. Previously, it was shown that SOCS3 disrupts TGF-β signaling in macrophages, via direct inhibition of the TGF-β signaling molecule Smad3 [3]. Inflammation in OA is known to disrupt TGF-β signaling in chondrocytes and additionally inhibits TGF-β-mediated cartilage repair. As SOCS3 is a pivotal regulator of inflammation, we investigated whether SOCS3 could be a key player in inflammation-induced obstruction of cartilage homeostasis via inhibition of TGF-β signaling. In contrast to our initial hypothesis and earlier findings in macrophages, our results in **chapter two** showed that SOCS3 did not alter TGF-β signaling in articular chondrocytes. We thoroughly investigated multiple aspects of the TGF-β signaling pathway, and did not find an effect of SOCS3 on TGF-β receptor expression, activation or nuclear translocation of Smad3, or TGF-β-mediated transcriptional activity in chondrocytes. Possibly, the effect of SOCS3 on TGF-β/Smad3 signaling is cell type-dependent, and may be present in other cell types such as macrophages [3]. Interestingly, SOCS3 did strongly impair MSC-based cartilage formation, a process which is also dependent on TGF-β. More specifically, overexpression of SOCS3 resulted in reduced pellet size and sGAG content. However, p-Smad3 levels in SOCS3-overexpressing MSCs were not different compared to luciferase control. Therefore, and based on our observations in chondrocytes, it is likely that SOCS3-dependent inhibition of MSC-based cartilage formation is mediated via a different mechanism which is independent of TGF-β. Both IGF-1 and STAT3 signaling pathways have been shown to stimulate cartilage formation [4-7], and are known targets of SOCS3 [8, 9]. Thus, SOCS3-mediated inhibition of MSC-based cartilage formation may therefore be caused by inhibition of IGF-1 or STAT3 signaling, which could be an interesting hypothesis for follow-up studies. Despite the lack of impact in mature chondrocytes,

based on our findings we suggest that targeting of SOCS3 in an inflammatory OA joint may improve MSC-based cartilage repair strategies. However, SOCS3 may also have an important protective role in the OA joint, as a key role for SOCS3 in controlling cytokine responses during (joint) inflammation has been demonstrated [9, 10]. Therefore care should be taken with this approach, as targeting of SOCS3 to improve MSC-based cartilage repair may lead to unwanted exacerbation of joint inflammation.

TGF-β restricts IL-6 signaling in chondrocytes via inhibition of IL-6R expression

Although inflammation-induced SOCS3 did not alter TGF-β signaling in chondrocytes, interplay between components of the IL-6 and TGF-β signaling pathway may occur via different mechanisms. In **chapter three** we reveal that in chondrocytes, TGF-β regulates IL-6 signaling on multiple levels. We first showed that exposure of human articular chondrocytes to TGF-β rapidly induced IL-6 expression and resulted in phosphorylation of STAT3, an IL-6R downstream signaling protein. To investigate if the p-STAT3 activation was caused by TGF-β-mediated IL-6 production, we stimulated chondrocytes with TGF-β in presence of the IL-6R blocking antibody tocilizumab. As expected, induction of p-STAT3 by TGF-β was completely prevented by blockade of the IL-6R, suggesting IL-6 dependency. Surprisingly, we observed no effect of TGF-β exposure on gene expression of the downstream STAT3-target gene *SOCS3*, while stimulation with exogenous IL-6 significantly increased *hSOCS3* expression. As we previously showed that exposure of chondrocytes to TGF-β ultimately also leads to IL-6 production, these results suggest that TGF-β potentially blocks IL-6-induced regulation of *hSOCS3* expression. To unravel the cause of this seemingly contradictory finding, we investigated if TGF-β could also block the effects of exogenous IL-6. We performed pre-treatment with TGF-β, mimicking the setting where cells are first exposed to TGF-β and afterwards to IL-6. Indeed, pre-treatment with TGF-β prevented IL-6-mediated induction of p-STAT3 and STAT3 target genes such as *hSOCS3*, *hBCL3*, *hMMP1* and *hSAA1*. These data demonstrate that TGF-β inhibits the IL-6 response in chondrocytes, despite upregulating IL-6 itself. In search for the potential underlying mechanism how TGF-β inhibits IL-6 signaling, we investigated whether TGF-β could potentially limit IL-6 signaling via interference with its receptor. Strikingly, we discovered that TGF-β exposure indeed resulted in a strong decrease in *hIL6R* expression. To confirm that TGF-β-mediated inhibition of IL-6 signaling was truly dependent on the IL-6R, we showed that addition of recombinant soluble IL-6R restored the TGF-β-mediated reduction of p-STAT3 and IL-6-repsonsive genes. With these experiments we uncovered a novel effect of TGF-β, which may play an important role in cartilage homeostasis by protecting cartilage via restriction of pro-inflammatory IL-6 effects. As a soluble form of the IL-6R is found in OA synovial fluid [11], this may bypass protective TGF-β-mediated inhibition of IL-6 signaling, rendering cartilage sensitive again for catabolic IL-6 effects. This highlights soluble IL-6R as a potential target for therapeutic strategies blocking IL-6 effects in OA.

Ageing negatively affects TGF-β-mediated suppression of IL-6R expression and signaling in articular cartilage

After we established that TGF-β regulated IL-6 signaling in cartilage, we hypothesized that ageing might affect this regulation as well, as previous studies of our lab showed that TGF-β signaling via Smad3 is reduced in ageing cartilage [12-15]. In **chapter four**, we therefore studied age-related changes in TGF-β-mediated regulation of IL-6 signaling. We collected cartilage from bovine MCP joints of 80 cows ranging from age 0.5-14 years old, which showed well-known characteristics of normal ageing, such as thinning of the cartilage surface, reduced chondrocyte numbers, and decreased expression of matrix components such as collagen type 2 and aggrecan [14, 16]. As expected, regression analysis showed that expression of *bIL6R* increased substantially with advancing age in articular cartilage (slope: 0.32, 95%CI: 0.20-0.45). In contrast, we observed no age-related changes in expression of *bGP130* clearly showing that this effect was IL-6R specific. To investigate if this change in IL-6R expression translated into the expected enhanced activation of intracellular signaling mediator STAT3, we exposed bovine cartilage of different ages to IL-6 *ex vivo*. This revealed that indeed induction of p-STAT3 in response to IL-6 was strongly increased upon ageing (slope: 0.14, 95%CI: 0.08 - 0.20). Furthermore, we showed that also IL-6-induced gene expression of catabolic target genes like *bMMP3*, *bPTGS2* and *bVEGFA* was increased in aged compared to young cartilage. Together, this confirmed that IL-6R expression and signaling was increased in bovine cartilage with advancing age. As we demonstrated in **chapter three** that TGF-β is an important regulator of IL-6R expression in articular cartilage, we hypothesized that age-related loss of TGF-β signaling might be the underlying cause for the increase in IL-6R levels. Stimulation with TGF-β indeed resulted in suppression of *bIL6R* in bovine cartilage, which was lost upon ageing (slope: 0.21, 95%CI: 0.13-0.30). Concurrently, we observed reduced TGF-β-mediated suppression of IL-6-induced p-STAT3 and *bSOCS3* expression in aged compared to young cartilage. Together, these observations provide proof that increased IL-6R expression and signaling in ageing cartilage can be explained by loss of TGF-β-mediated IL-6R suppression. Due to the catabolic and pro-inflammatory role of IL-6 in OA development, this implies that TGF-β-mediated loss of IL-6R suppression with age predisposes cartilage to degenerative changes, ultimately contributing to age-related OA development.

The IL-6 signaling pathway as a therapeutic target in OA

In **chapter three** and **four** we established an important role of TGF-β in dampening IL-6 signaling in articular cartilage, which might be lost during ageing or inflammation. These implications point towards the IL-6 signaling pathway as a potential target in OA. As IL-6 has the unique ability to signal via a classic- and trans-signaling pathway, this may have implications for future therapy development. In chapter **five**, we discussed the opposing effects of IL-6 classic versus trans signaling in OA pathophysiology and provided future perspective for OA treatment. We reported that IL-6 classic signaling mainly results in protective effects in local joint tissues such as cartilage, synovium and bone, while trans-signaling leads to pro-inflammatory and catabolic effects. For

example, IL-6 trans signaling more strongly activates the expression of cartilage degenerative enzymes, while classic signaling has also been linked to protection against matrix degradation. Moreover, we described that local regulation of IL-6R levels e.g. by hormones, cytokines and epigenetic factors also determines IL-6 outcome to a great extent. Examination of previous studies that block IL-6 in experimental OA show that therapeutic targeting of the IL-6 pathway could be a promising treatment strategy to reduce cartilage damage, synovial inflammation and subchondral bone pathology. However, all of the employed blocking strategies block both the classic- and trans signaling pathway. We argue that specific inhibition of IL6 trans signaling in OA models might be extremely helpful to dissect detrimental versus protective effects of IL-6 in the future. In other inflammatory diseases, specific blockade of IL-6 trans signaling using soluble gp130 resulted in the blockade of pro-inflammatory events of IL-6 trans signaling, while simultaneously allowing homeostatic effects of IL-6 classic signaling. We argue that this approach could also be a preferred treatment strategy for OA patients, as several studies demonstrated catabolic and proinflammatory effects of IL-6 trans signaling in cartilage and synovial tissue. Nonetheless, additional pre-clinical research will first be needed as the significance of IL-6 trans signaling in experimental OA has not yet been demonstrated.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In this thesis we have studied two potential crosstalk mechanisms between TGF-β and IL-6 signaling in articular cartilage. In Figure 1 below, our findings are summarized in a schematic representation. Interplay between IL-6 and TGF-β can occur via two routes: 1) regulation of TGF-β signaling by IL-6, or 2) regulation of IL-6 signaling by TGF-β. With regard to the first route, we showed that SOCS3, as important negative regulator of the IL-6 signaling pathway, does not inhibit TGF-β/Smad3 signaling in chondrocytes (**chapter two**). With regard to the second route, we demonstrated that TGF-β has an important role in regulating the IL-6 pathway by suppression of IL-6R expression and signaling in chondrocytes (**chapter three**). Of course this merely represents a small selection of the potential mechanisms of interplay between the TGF-β and IL-6 signaling pathways. For instance, it still remains to be investigated whether IL-6 regulates TGF-β (receptor) expression and signaling or whether Smad3/STAT3 interact in chondrocytes. Also, as reviewed in **chapter five**, it could be important to compare the effects of IL-6 classic- versus trans signaling when studying interplay with TGF-β signaling in cartilage. Finally, we show that the (patho)physiological context can alter TGF-β and IL-6 crosstalk, such as older age or OA-related inflammation as demonstrated in **chapter three**, **four** and **five** of this thesis.

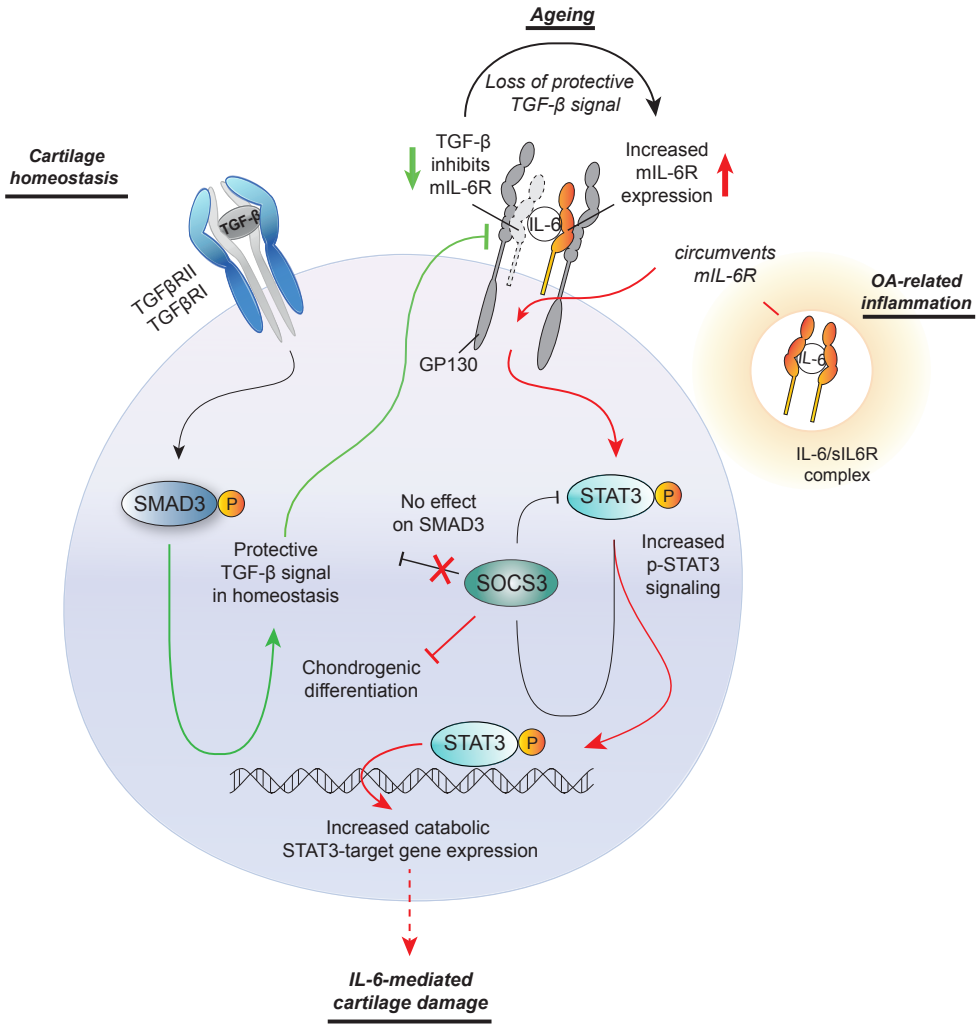

Figure 1. Schematic representation of observed interplay between the TGF-β and IL-6 signaling pathways as observed in chapter 2, 3 and 4 of this thesis. Protective regulation indicated in green, detrimental regulation indicated in red. mIL-6R: membrane-bound IL-6R, sIL-6R: soluble IL-6R.

When comparing our findings with published literature, it became clear that crosstalk between TGF-β and IL-6 signaling might be strongly cell- or tissue dependent. In T-cell differentiation, for example, IL-6 and TGF-β have synergistic effects to stimulate the development of Th17 cells [17, 18]. It has been demonstrated that TGF-β increases IL-6R expression in naive T-cells which promotes Th17 cell differentiation [17]. Moreover, TGF-β inhibits SOCS3 expression resulting in enhanced STAT3 activation which further stimulates Th17 cell development [18]. In the context of liver fibrosis on the other hand, TGF-β and IL-6 have an antagonistic relationship. In the liver,

IL-6 has an important anti-inflammatory and regenerative role [19], while TGF-β stimulates liver fibrosis [20]. Indeed, it has been shown that SOCS3 has a protective role during liver fibrosis by inhibiting STAT3-mediated TGF-β production [21]. In articular cartilage, IL-6 and TGF-β are thought to have opposite effects, but whether they have a synergistic or antagonistic relationship was yet unclear. IL-6 has a detrimental role in cartilage degeneration [22-24], while TGF-β has a protective role in cartilage homeostasis [16, 25, 26], which suggests that interplay between TGF-β and IL-6 in articular cartilage may result in antagonism. Indeed, in **chapter three** we demonstrate that TGF-β dampens IL-6 signaling via suppression of IL-6R expression in articular cartilage, which may be important for the maintenance of cartilage homeostasis. Altogether, this suggests that the result of interplay between TGF-β and IL-6 is cell- and context dependent, and may depend on the role of these factors in the respective tissue. Indeed, this is a likely conclusion as studies show that TGF-β and IL-6 signaling have different roles and impressive versatility during context-specific cellular behavior, and that this largely depends on crosstalk with other signaling pathways [27-29]. Below we discuss the implications of our mechanistic findings in relation to cartilage biology and the development of therapeutic strategies for OA patients.

Although we demonstrated in **chapter two** that SOCS3, as negative regulator of IL-6 signaling does not affect TGF-β/Smad3 signaling in articular chondrocytes, a detrimental role for SOCS3 during MSC-based cartilage formation was identified. This suggests that targeting of SOCS3 could be a therapeutic strategy to improve cartilage repair in OA. However, SOCS3 is crucial for restriction of pro-inflammatory cytokine signaling [9, 30], suggesting that targeting of SOCS3 in OA patients may lead to exacerbated inflammation and cartilage damage, instead of improved cartilage repair. Indeed, deletion of SOCS3 in murine articular cartilage resulted in increased cartilage degradation, increased joint inflammation and enhanced expression of catabolic mediators after intra-articular injection of the IL-6/sIL-6R complex [10]. Furthermore, at present there are no inhibitors in the clinic that specifically target SOCS3. Potentially, it would be easier to directly target IL-6 itself to prevent upregulation of SOCS3. However, there are several other cytokines present in the OA environment which also induce SOCS3 expression via gp130-JAK/STAT signaling, such as Oncostatin M (OSM), Leukemia Inhibitory Factor (LIF) and IL-11 [1, 31, 32]. Moreover it may even be disadvantageous to inhibit IL-6 to promote cartilage repair as there are some indications that IL-6 signaling may stimulate cartilage regeneration [4, 5]. In an *in vitro* cartilage regeneration model, addition of IL-6 resulted in increased proteoglycan production by healthy chondrocytes and decreased proteoglycan release by OA chondrocytes [5]. Moreover, chondrogenic differentiation was positively stimulated after addition of IL-6 and sIL-6R, resulting in increased matrix generation and cartilage marker gene expression [4]. Thus, *in vivo* targeting of IL-6 or SOCS3 to improve MSC-based cartilage repair is not yet desirable. First, it is important to eliminate the possibility that targeting of SOCS3 in the OA joint leads to exacerbated inflammation or damage.

In this thesis, we uncovered a novel function of TGF-β in articular cartilage. We showed that TGF-β was able to dampen IL-6 signaling in cartilage via suppressing IL-6R expression. Cartilage contains large amounts of inactive TGF-β, which can be activated upon loading [33, 34]. Via this mechanism, TGF-β maintains cartilage homeostasis in physiological circumstances, e.g. by preventing hypertrophic differentiation of chondrocytes [33, 35]. Possibly, TGF-β mediated down regulation of IL-6R is also important for homeostatic regulation of articular cartilage by protecting against catabolic IL-6 signaling [33, 36]. In **chapter three** and **four**, we identified two possible mechanisms how this protective TGF-β effect might be dysregulated during OA. First, we showed that soluble IL-6R can bypass TGF-β-mediated IL-6R suppression, rendering cartilage sensitive again for catabolic IL-6 signaling. Secondly, we demonstrated that ageing negatively affects TGF-β-mediated IL-6R suppression in cartilage, resulting in increased IL-6 signaling with advancing age (illustrated in Fig 2. of this chapter). These mechanisms shed new light on how changes in chondrocyte signaling during ageing or inflammation can contribute to IL-6-mediated cartilage degeneration and OA progression.

As mentioned earlier, soluble IL-6R may be able to bypass TGF-β-mediated IL-6R suppression, rendering cartilage sensitive again for catabolic IL-6 signaling. This mechanism is dependent on the levels and availability of sIL-6R in OA patients. As described in **chapter five**, sIL-6R has already been detected in OA serum and synovial fluid [37-39], and may even be enhanced in a subset of OA patients. Production of sIL-6R is regulated by shedding of membrane-bound IL-6R or via differential splicing of IL-6R mRNA [40-42]. Shedding of membrane IL-6R is largely regulated by the ADAM class of metzincin proteases, of which ADAM17 (or TACE) and ADAM10 are identified as important proteases for cleavage of the IL-6R [43, 44]. However, whether these proteases are also involved in generation of sIL-6R in OA patients is unknown. It has been suggested that ADAM17 activity may be enhanced in OA patients in relation to a rare variant of secreted Frizzled-related protein 3 (sFRP3) [45]. As ADAM17 mediates IL-6R shedding, this mechanism may contribute to sIL-6R production in OA. sFRP3 acts as an antagonist of Wnt signaling, by delaying hypertrophic chondrocyte differentiation and rare variants of sFRP3 have been associated with OA development [46, 47]. sFRP3 was also identified as a novel suppressor of ADAM17 activity, which resulted in inhibition of ADAM17-mediated IL-6R shedding [45]. Unfortunately, this study did not confirm whether ADAM17 activity and subsequent sIL-6R production were increased in cartilage of OA patients compared to healthy individuals. Besides shedding of membrane IL-6R, differential mRNA splicing also contributes to sIL-6R production [42]. Differential mRNA splicing generates two distinct IL-6 mRNA transcripts: one encoding sIL-6R and the other encoding the cognate membrane-bound IL-6R. The first variant lacks the coding sequence for the transmembrane domain, which results in a reading frameshift and the introduction of a unique COOH-terminal sequence at the tail of this IL-6R isoform [48]. It is not clear yet whether alternative splicing of IL-6R also contributes to generation of sIL-6R in OA. One study

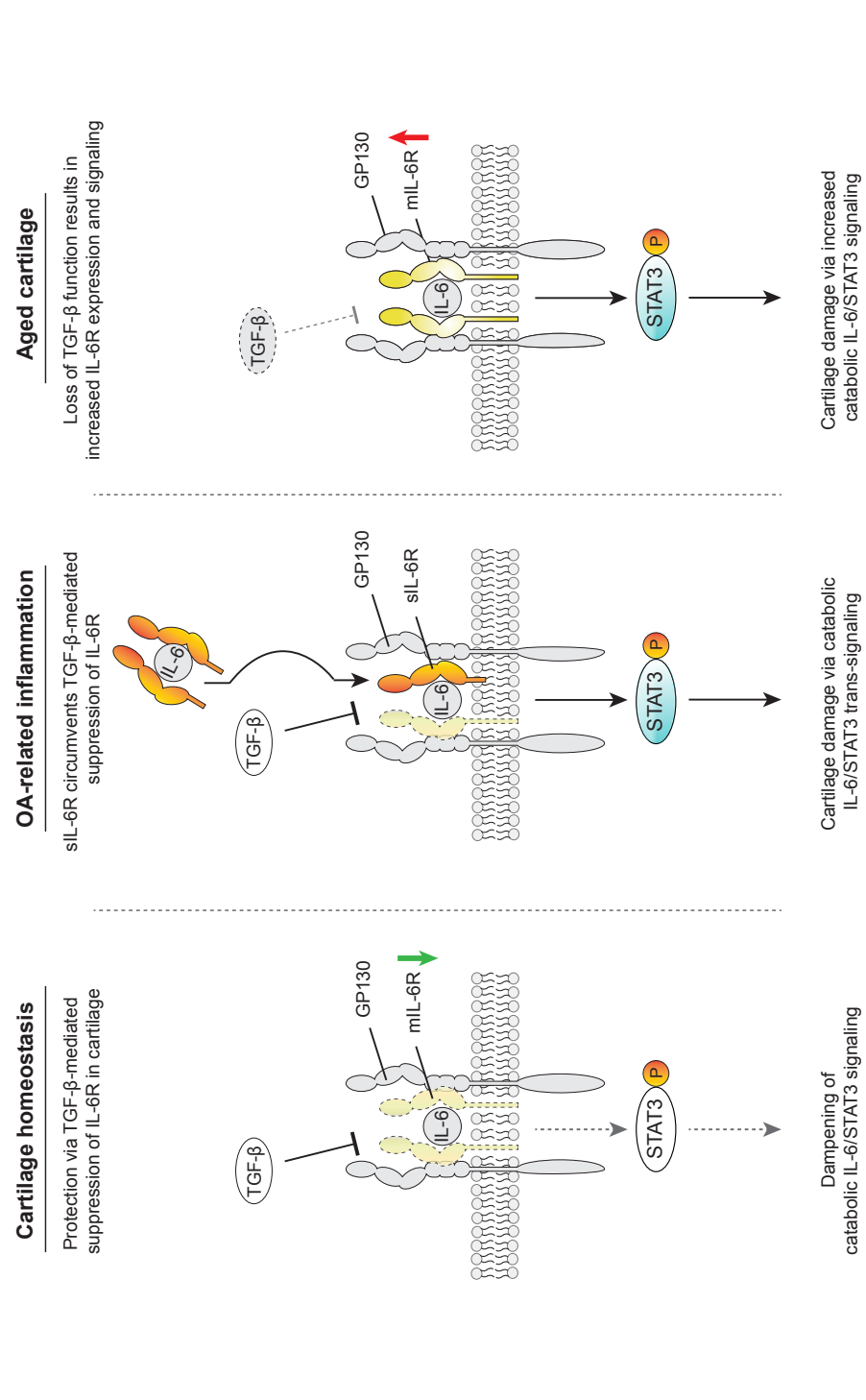

Figure 2. Proposed regulation of IL-6 signaling by TGF-β during cartilage homeostasis, ageing or inflammatory OA.

screened synovial fluid of 15 OA patients, using an antibody directed against the COOH-terminal sequence of sIL-6R, but found only isoforms of differentially spliced sIL-6R in RA patients [49].

Interestingly, levels of sIL-6R are increased up to three-fold in numerous inflammatory diseases [50, 51]. As synovitis is observed in around 50% of the OA patients [52-57], it is possible that sIL-6R levels are especially increased in inflammatory OA patients. Unfortunately, studies which investigate sIL-6R levels in different subsets of OA patients are lacking. Moreover, it is necessary to compare sIL-6R levels in OA patients to healthy individuals, to gather evidence that sIL-6R is increased during disease and may be relevant in the disease process. As the availability of sIL-6R in the joint is also determined by the presence of its natural antagonist sgp130 [58], it will be crucial to investigate levels of sgp130 in OA patients as well. Thus, in order to demonstrate that sIL-6R mediates cartilage degeneration in OA patients by circumventing TGF-β suppression of membrane IL-6R in chondrocytes, future studies first need to confirm that sIL-6R is indeed increasingly available in (a subset) of OA patients and contributes to OA pathology. This would identify sIL-6R as a potential target in OA patients, which could be achieved using a fusion protein consisting of two soluble human gp130 proteins fused with the Fc region of human IgG (sgp130Fc) [59]. This fusion protein was already successfully used in treating experimental Crohn's disease, is now in clinical trials for inflammatory bowel disease and active ulcerative colitis [60, 61] and may also hold promise for OA treatment.

The data in chapter **three** (illustrated in Figure 2) demonstrate that TGF-β inhibits the IL-6 response in articular chondrocytes, despite upregulating IL-6 itself. However, nowadays OA is viewed as a "whole joint" disease, with pathophysiological hallmarks such as subchondral bone changes, osteophyte formation, inflammation and synovial fibrosis [62, 63]. It is possible that TGF-β-induced IL-6 production does not activate chondrocytes due to decreased IL-6R levels (as shown in **chapter three**), but does activate cells in other joint tissues such as synovium or bone. However, as reviewed in **chapter five**, the exact role of IL-6 in (healthy) synovium and bone is still unclear making it difficult to interpret the implications of this hypothesis. Alternatively, TGF-β may also regulate IL-6R expression in tissues other than articular cartilage. Therefore, it would be interesting to investigate whether TGF-β also suppresses IL-6R expression in other (joint) tissues or whether this mechanism is unique for articular cartilage.

In **chapter four** we described the identification of a second mechanism which bypasses protective TGF-β-mediated suppression of IL-6R in chondrocytes and therefore may contribute to cartilage damage in OA (Figure 2). We established that ageing leads to loss of TGF-β-mediated IL-6R suppression, thus resulting in increased IL-6 signaling in cartilage. As systemic IL-6 levels highly increase in ageing individuals [64], this mechanism may be involved in the development of age-related OA due to detrimental IL-6 effects in cartilage. We expect that the observed loss in TGF-β-mediated IL-6R expression is caused by decreased expression of ALK5 in aged cartilage. Our

lab has previously demonstrated that ALK5 expression strongly decreases with advancing age in bovine articular cartilage, while ALK1 expression is unaffected [14]. Moreover, we observed an increase in the ratio of ALK1/ALK5 expression in murine cartilage upon ageing, which correlates with OA development [13]. In addition, ageing also results in reduced signaling via the protective Smad2/3 pathway [12, 14, 16, 65], a pathway which is activated via the ALK5 receptor [66, 67]. Interestingly, Smad2 directly regulates IL-6R expression in T-cells [68], supporting our hypothesis that TGF-β suppresses IL-6R expression in chondrocytes via the ALK5-Smad2/3 signaling route. We did not investigate whether TGF-β-induced IL-6 production by chondrocytes, as observed in **chapter three**, was affected upon ageing. As TGF-β signaling decreases with ageing, diminished TGF-β-induced IL-6 production is expected with older age. However, systemic IL-6 levels are increased with older age [64] and IL-6 is spontaneously produced by senescent chondrocytes [64]. It is therefore likely that sufficient IL-6 is present to activate IL-6 signaling in articular chondrocytes. Furthermore, it is plausible that miRNA's are involved as well in TGF-β-mediated suppression of IL-6R, as epigenetic changes are a hallmark of human ageing, and the expression of several miRNA's is altered in aged cartilage [69, 70]. For example, expression of miR-34 is related to cell senescence and mitochondrial dysfunction which also plays a role in age-related cartilage degeneration [64, 71-73]. Moreover, TGF-β can modulate expression of miR-34a [73], which suggests that altered expression of miR-34a in ageing cartilage may contribute to loss of TGF-β-mediated IL-6R suppression and the development of chondrocyte senescence and mitochondrial dysfunction. Our observations indicate that reverting or inhibiting the age-related increase in IL-6R expression in chondrocytes will be a future challenge to combat age-related OA development, for instance by restoring the ALK5-Smad2/3 signaling balance. Alternatively, therapies for OA could focus on neutralizing the increase in IL-6 levels observed with ageing [64] or upon joint injury [5, 74], in order to prevent activation of highly expressed IL-6R in aged cartilage. It could be very relevant to study gender-related differences in this context as well, as IL-6 has been found to have different effects in males compared to females in relation to cartilage damage and OA development [75].

IL-6 has the unique ability two signal via two distinct signaling pathways which is determined by activation of either membrane-bound IL-6R (classic signaling) or soluble IL-6R (trans signaling). In **chapter three** we identified a novel mechanism via which TGF-β restricts IL-6 classic signaling by suppressing membrane-bound IL-6R expression. Moreover, in **chapter four** we showed that ageing results in increased IL-6 classic signaling in cartilage, which is probably caused by an age-related increase in membrane-bound IL-6R expression. Although our results show that IL-6 classic signaling increases the expression of catabolic mediators in chondrocytes (e.g. MMP-1, MMP-3, COX-2, VEGF-A) we did not extensively investigate the long-term effects on cartilage matrix synthesis or degeneration. As also protective effects of IL-6 classic signaling in cartilage have been demonstrated [5, 76, 77], additional studies are needed that investigate the long-term effects of TGF-β-mediated regulation of IL-6 classic signaling on cartilage homeostasis to truly

conclude whether this mechanisms is protective. As so far the role of IL-6 in direct modulation of proteoglycan synthesis or degeneration is modest [5, 78-81], detrimental IL-6 signaling may additionally play a role in other pathological processes in OA. Interestingly, there are indications that IL-6 signaling is involved in the development of chondrocyte hypertrophy [82, 83], pre-mature senescence [84, 85] or the dysregulation of chondrocyte energy metabolism [86, 87]. It may be crucial to study the role of IL-6 in these processes in the future, in order to completely define IL-6 function in cartilage. Furthermore, we suggest in **chapter five** that functional differences in IL-6 classic- versus trans signaling may account for some of the protective and degenerative effects of IL-6 in cartilage. This may be of particular importance, as we demonstrate in **chapter three** that the presence of soluble IL-6R can bypass TGF-β-mediated suppression of mIL-6R, leading to activation of the IL-6 trans signaling pathway. Several *in vitro* studies indeed demonstrated catabolic and pro-inflammatory effects of IL-6 trans signaling in chondrocytes [22, 88-90], while classic signaling has been linked to protective effects such as induction of anti-catabolic TIMPs [76, 77]. However, this distinction is not black and white as, for example, IL-6 trans signaling has also been reported to increase the production of TIMPs [76]. Moreover, up until now there is no direct proof that implicates IL-6 trans signaling in OA development. Therefore, specific inhibition of IL-6 trans signaling in OA models is needed to dissect functional differences between IL-6 classic- versus trans signaling in OA development. Possibly, both the activation of IL-6 trans signaling in OA, as well as disturbed expression of mIL-6R levels on chondrocytes contributes to IL-6-mediated cartilage degeneration in OA patients.

In this thesis we describe novel interactions between IL-6 and TGF-β signaling in articular cartilage, which may be very relevant for cartilage homeostasis and OA development. We provide a strong foundation for future fundamental and clinical studies to inhibit IL-6 signaling in OA, and identify sIL-6R as a potential therapeutic target.

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kan in één gewricht voorkomen zoal
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j afbraak van het kraakbeen, zoal
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Chapter 7

Nederlandse samenvatting

Curriculum Vitae

List of publications

PhD Portfolio

Research data management

Dankwoord

NEDERLANDSE SAMENVATTING

Artrose

Artrose is de meest voorkomende aandoening aan het bewegingsapparaat. Meer dan 1 miljoen mensen in Nederland lijden op dit moment aan deze chronische gewrichtsaandoening, en er wordt verwacht dat dit aantal aanzienlijk toe zal nemen in de toekomst onder andere door verdere vergrijzing van de bevolking. Er is niet één duidelijke oorzaak te benoemen voor artrose, maar meerdere risicofactoren verhogen de kans op ontwikkeling van deze ziekte. Belangrijke risicofactoren zijn erfelijke aanleg, ouderdom, het vrouwelijke geslacht, overgewicht en gewrichtstrauma. Een belangrijk kenmerk van artrose is dat de kwaliteit van de kraakbeen laag op de gewrichtsuiteinden zodanig verminderd dat dit resulteert in functieverlies. Het gladde oppervlak zorgt ervoor dat de gewrichtsuiteinden soepel langs elkaar heen kunnen bewegen. Tevens heeft het kraakbeen een essentiële functie in het gewricht door middel van het dempen van belasting die ontstaat door beweging. Bij afbraak van het kraakbeen, zoals tijdens artrose, ontstaat er uiteindelijk direct bot op bot contact wat gepaard gaat met pijn en bewegingsbeperking. Naast schade aan het kraakbeen, ontstaan er andere veranderingen in het gewricht. Zo neemt de dichtheid van het onderliggende (subchondrale) bot toe, en daarnaast ontwikkelen er abnormale botknobbels aan de rand van het gewricht. Artrose kan in één gewricht voorkomen zoals alleen in de knie, heup of duim, maar ook in meerdere gewrichten tegelijkertijd. Veel voorkomende klachten zijn stijfheid en pijn. Huidige therapieën richten zich op symptoombestrijding, zoals het bestrijden van pijn en verbetering van levensstijl door meer beweging en gewichtsverlies. Tevens kan bij vergevorderde artrose een gewricht worden vervangen door een kunstgewricht. Pijnklachten zijn echter niet altijd verholpen door een gewrichtsvervanging, en de kunstgewrichten hebben een beperkte levensduur van gemiddeld 15 jaar. Op dit moment kan artrose niet worden genezen en hierdoor is er een grote behoefte aan een therapie die het ziekteproces remt. Voor de ontwikkeling van deze therapieën is het uiterst belangrijk om te begrijpen hoe kraakbeen zich gedraagt in een gezond gewricht en waarom er schade ontstaat tijdens de ontwikkeling van artrose.

Artrose en gewrichtskraakbeen

Gewrichtskraakbeen, ook wel articulair kraakbeen genoemd, bestaat uit kraakbeencellen omringd door extracellulaire matrix die stevigheid en structuur biedt. Matrixmoleculen aanwezig in de extracellulaire matrix, zoals aggrecan en collageen type II, zijn belangrijk voor de functie van het kraakbeen als schokdemper. Kraakbeencellen zijn essentieel voor onderhoud van een gezonde kraakbeenmatrix. Onder andere door een balans te onderhouden tussen afbraak van kraakbeen middels kraakbeenafbrekende enzymen, en de aanmaak van nieuwe matrixmoleculen. In gezond kraakbeen, is er een balans tussen de opbouwende (= anabole) en afbrekende (= katabole) processen. Tijdens artrose is deze balans echter verstoord, resulterend in een verschuiving naar kraakbeenafbraak. Katabole processen tijdens artrose worden onder andere gereguleerd

door ontstekingsmediatoren. Zelfs bij milde artrose speelt een lichte, chronische ontsteking in het gewricht een belangrijke rol. Artrose is niet te vergelijken met ontstekingsreuma; een auto-immuunziekte waarbij hevige, chronische ontsteking optreedt in meerdere gewrichten. Bij artrose wordt met name ontsteking in de slijmvlieslaag (synovium) van het gewricht vastgesteld in zeker 50% van de artrose patiënten. Het remmen van ontstekingsfactoren tijdens artrose is dus mogelijk een belangrijk aangrijpingspunt voor de ontwikkeling van toekomstige therapieën.

De rol van groeifactor TGF-β in gezond en verouderd kraakbeen

Zoals eerdergenoemd, is er in een balans tussen de opbouwende (= anabole) en afbrekende (= katabole) functies van kraakbeencellen in gezond kraakbeen. Deze balans wordt onder andere gereguleerd door signaalmoleculen buiten de cel, die kraakbeenopbouw of afbraak stimuleren. Een belangrijke signaalmolecuul die kraakbeen gezond houdt, is de groeifactor TGF-β (= transforming growth factor-β). TGF-β stimuleert bijvoorbeeld de productie van extracellulaire kraakbeen matrix en heeft een belangrijke ontstekingsremmende werking in kraakbeen. Echter is deze beschermende werking van TGF-β verstoord tijdens artrose, en kan TGF-β hier zelfs een schadelijke rol hebben in het gewricht. TGF-β kan via twee verschillende signaalroutes kraakbeencellen activeren, die gebruik maken van een andere receptor genaamd ALK5 en ALK1. Binding van TGF-β aan deze receptoren resulteert in de activatie van intracellulaire Smad-moleculen. Eerder onderzoek heeft aangetoond dat binding van TGF-β aan de ALK5 receptor voornamelijk leidt tot activatie van de Smad2/3 signaalroute. Binding van TGF-β aan de ALK1 receptor leidt tot activatie van de Smad1/5/9 route. In gezond kraakbeen wordt voornamelijk de ALK5-Smad2/3 signaalroute geactiveerd, welke kraakbeenopbouwende processen stimuleert en een beschermende werking heeft op kraakbeen. Tijdens artrose is de hoeveelheid ALK5 echter verlaagd op de kraakbeencellen, wat leidt tot verminderde activatie van de Smad2/3 signaal route waardoor de ALK1-Smad1/5/9 signaalroute de overhand heeft. De ALK1-Smad1/5/9 route is echter betrokken bij kraakbeenafbrekende processen zoals de productie van kraakbeenafbrekende enzymen. Eerder onderzoek heeft aangetoond dat ook tijdens veroudering de beschermende ALK5-Smad2/3 signaalroute van TGF-β verminderd werkzaam is in kraakbeen, wat mogelijk bijdraagt aan het ontstaan van leeftijdgerelateerde artrose. Door te onderzoeken wat de oorzaak is van de verstoorde werking van TGF-β tijdens veroudering en artrose kunnen mogelijk in de beschermende en schadelijke effecten van TGF-β van elkaar gescheiden worden in de toekomst. Deze kennis zou kunnen leiden tot de ontwikkeling van therapieën die kraakbeenschade remmen of voorkomen. Zoals eerdergenoemd speelt ontsteking een belangrijke rol in het ontstaan van kraakbeenschade en artrose. In kraakbeen leidt de aanwezigheid van ontstekingsmediatoren bijvoorbeeld tot stimulatie van kraakbeenafbrekende processen. Er zijn aanwijzingen dat de aanwezigheid van ontstekingsmediatoren tijdens artrose de signaalroute van TGF-β kunnen verstoren. In dit proefschrift onderzoeken we de interactie tussen de groeifactor TGF-β en de ontstekingsmediator interleukin-6 (IL-6).

Interleukine-6

IL-6 is een ontstekingsmediator die in hoge spiegels aanwezig is in de gewrichtsvloeistof van patiënten met artrose. Tevens zijn verhoogde waarden van IL-6 in het bloed geassocieerd met een verhoogde kans op artrose. Dit suggereert dat IL-6 een belangrijke speler kan zijn in het ontstaan van artrose. De IL-6 signaalroute wordt geactiveerd door binding van IL-6 aan de IL-6 receptor (IL-6R) op de cel, waarna de intracellulaire mediator STAT3 wordt geactiveerd. Deze IL-6R is echter niet aanwezig op elk celtype, waardoor gevoeligheid voor IL-6 lokaal gereguleerd kan worden. Eerder onderzoek laat zien dat de IL-6R in lage niveaus aanwezig is op kraakbeencellen, maar dat het expressieniveau verhoogd wordt door onder andere epi genetische regulatie of door ontsteking. Een belangrijke remmer van de IL-6 signaalroute is het eiwit suppressor of cytokine signaling 3 (SOCS3). SOCS3 wordt snel geactiveerd door IL-6, en zorgt voor negatieve terugkoppeling via de remming van intracellulaire mediator STAT3. In het kraakbeen van artrose patiënten is SOCS3 verhoogd aanwezig, wat mogelijk duidt op sterke activatie van de IL-6 signaalroute. IL-6 heeft een schadelijke rol in kraakbeen en stimuleert kraakbeenafbrekende processen. *In vitro* experimenten laten zien dat stimulatie van kraakbeencellen met IL-6 resulteert in verhoogde productie van kraakbeenafbrekende enzymen, zoals matrix metalloproteinases (MMPs) en A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) enzymen. *In vivo* studies bevestigen deze schadelijke rol van IL-6 in kraakbeen. Injectie van IL-6 in het kniegewricht van de muis resulteert in kraakbeenschade en verhoogde MM-3 en MMP-13 expressie. Therapeutische remming van IL-6 tijdens experimentele artrose in muismodellen vermindert kraakbeenschade en synoviale ontsteking. Dit suggereert dat IL-6 mogelijk een belangrijke rol speelt in het ontstaan van kraakbeenschade tijdens artrose.

Doel van dit proefschrift

Ondanks dat zowel TGF-β als IL-6 cruciale processen reguleren in articulair kraakbeen, is het nog onbekend of deze twee signaalmoleculen elkaar beïnvloeden. IL-6 heeft een schadelijke rol in kraakbeen terwijl TGF-β juist beschermend werkt. Het is mogelijk dat interactie tussen TGF-β en IL-6 deze rollen beïnvloedt, resulterend in minder of juist meer kraakbeenschade. TGF-β heeft bijvoorbeeld een belangrijke ontstekingsremmende functie in kraakbeen, en eerdere studies hebben aangetoond dat schadelijke effecten van ontstekingsmediatoren zoals TNF-α en IL-1β geremd worden door TGF-β. Het is echter nog niet onderzocht of TGF-β ook de schadelijke effecten van IL-6 in kraakbeen kan remmen. Daarnaast is het bekend dat ontsteking kan leiden tot verstoring van de beschermende werking van TGF-β in kraakbeen, maar of IL-6 betrokken is bij verstoring van de TGF-β signaalroute tijdens artrose is nog onbekend. Een eerdere studie laat zien dat het eiwit SOCS3 de signaalroute van TGF-β verstoort in afweercellen, via remming van Smad3. Aangezien SOCS3 sterk wordt geactiveerd door IL-6, en beide IL-6 en SOCS3 verhoogd aanwezig zijn tijdens artrose, is het mogelijk dat SOCS3 de TGF-β signaalroute verstoort in kraakbeen van artrose patiënten. In dit proefschrift hebben we deze potentiële interacties tussen de signaalroutes van TGF-β en IL-6 bestudeerd in articulair kraakbeen, en

de implicaties hiervan bestudeerd tijdens veroudering en artrose. Door te onderzoeken welk samenspel van factoren kraakbeen gezond houdt, en hoe dit verstoord is tijdens artrose, kunnen er nieuwe aangrijpingspunten voor therapie worden ontwikkeld met als doel het remmen of zelfs voorkomen van kraakbeenschade.

SOCS3 heeft geen remmend effect op TGF-β signaalroute in kraakbeen maar verstoort mogelijk kraakbeenherstel

Zoals vermeld kunnen ontstekingsfactoren de werking van TGF-β verstoren wat mogelijk bijdraagt aan de ontwikkeling van kraakbeenschade. Een centrale factor die aanwezig is tijdens ontstekingsprocessen is het eiwit SOCS3 dat sterk geactiveerd wordt door aanwezigheid van IL-6. Het is eerder aangetoond dat SOCS3 de TGF-β signaalroute verstoord in andere celtypen (macrofagen), door de belangrijkste signaalmolecuul van de TGF-β signaalroute, Smad3 te remmen. Het is eerder bewezen dat SOCS3 verhoogd aanwezig is in kraakbeen en synovium tijdens artrose. In **hoofdstuk 1** hebben we onderzocht of deze verhoogde expressie van SOCS3 tijdens gewrichtsontsteking kan leiden tot verstoring van TGF-β signaalroute en functie in articulair kraakbeen. Dit zou een mogelijke verklaring zou kunnen zijn voor ontwikkeling van kraakbeenschade tijdens artrose. We hebben dit onderzocht door de eiwitproductie van SOCS3 artificieel te verhogen in kraakbeencellen, en vervolgens meerdere componenten van de TGF-β signaalroute te onderzoeken zoals de expressie van de TGF-β receptoren en de activatie van Smad-moleculen in de cel. In tegenstelling tot de eerdere bevindingen, zoals die in macrofagen, laten onze resultaten geen effect zien van SOCS3 op de TGF-β signaalroute in kraakbeen. Dit suggereert dat het effect van SOCS3 op de TGF-β signaalroute mogelijk verschilt per celtype.

Naast het effect van SOCS3 op de signaalroute van TGF-β, hebben we ook de rol van SOCS3 in kraakbeenvorming getest. Om het beschadigde kraakbeen van artrose patiënten te herstellen kan gebruik gemaakt worden van mesenchymale stamcellen. Deze stamcellen zijn in staat om nieuw kraakbeen te vormen onder bepaalde omstandigheden, bijvoorbeeld door de toevoeging van de groeifactor TGF-β. De ontstekingsomgeving van een artrotisch gewricht heeft echter een remmende werking op deze nieuwe kraakbeenvorming, wat de toepassing van deze techniek belemmert. In theorie zou verhoogde expressie van SOCS3 tijdens artrose de vorming van nieuw kraakbeen dus kunnen remmen. Om dit aan te tonen hebben we de SOCS3 eiwitproductie verhoogd in mesenchymale stamcellen en vervolgens deze cellen geweekt in een driedimensionaal (3D)-pellet model. Dit is een geschikt model om kraakbeenvorming te bestuderen onder invloed van de groeifactoren TGF-β en BMP-2, gedurende een periode van 7 dagen. Verhoogde expressie van SOCS3 resulteerde in een sterke remming van de kraakbeenvorming. Stamcelpellets met verhoogde SOCS3 niveaus waren duidelijk kleiner en vertoonde significant minder extracellulaire kraakbeen matrix dan stamcelpellets met het controle eiwit. De activatie van het TGF-β signaalmolecuul Smad3 in de stamcellen was echter hetzelfde in beide condities. Dit suggereert dat de remming van kraakbeenvorming door SOCS3

onafhankelijk is van TGF-β, maar voortkomt uit een ander mechanisme. Een mogelijke kandidaat is de signaalmolecuul IGF-1. IGF-1 is cruciaal voor de aanmaak van kraakbeenbouwstenen zoals glycosaminoglycanen. Tevens is het bekend dat SOCS3 de IGF-1 signaalroute remt in articulair kraakbeen. Mogelijke vervolgstudies moeten uitwijzen of verminderde kraakbeenvorming door SOCS3 verklaard kan worden door remming van de IGF-1 signaalroute. Deze resultaten suggereren dat de remming van SOCS3 in een artrotisch gewricht zou kunnen leiden tot beter herstel van kraakbeenschade tijdens stamceltherapie. Echter heeft SOCS3 ook een potentiële beschermende rol in het artrotisch gewricht, als negatieve regulator van ontstekingscytokines zoals IL-6. Mogelijk is het therapeutisch remmen van SOCS3 daardoor niet wenselijk, omdat het kan leiden tot verergerde gewrichtsontsteking en schade.

TGF-β remt IL-6 signaalroute in kraakbeen via het verlagen van de IL-6 receptor

Interactie tussen de TGF-β en IL-6 signaalroutes kan plaatsvinden via verschillende mechanismen. In andere celtypen is beschreven dat TGF-β effecten van IL-6 kan remmen, maar of dit ook plaatsvindt in kraakbeen is nog onbekend. Aangezien IL-6 een schadelijk effect heeft in kraakbeen, zou dit een mogelijk beschermend mechanisme kunnen zijn. In **hoofdstuk 3** beschrijven we dat TGF-β de IL-6 signaalroute op meerdere manieren beïnvloedt in humane kraakbeencellen. Ten eerste hebben we aangetoond dat het toevoegen van TGF-β aan kraakbeencellen leidt tot productie van IL-6 en activatie van de intracellulaire mediator STAT3. Dit suggereert dat TGF-β de IL-6 signaalroute mogelijk stimuleert in kraakbeencellen. Dit was een verrassende bevinding, omdat TGF-β juist een aangetoonde ontstekingsremmende werking heeft in kraakbeen. Om te onderzoeken of activatie van STAT3 ook leidde tot de activatie van genexpressie, hebben we de expressie van het STAT3-afhankelijke gen SOCS3 gemeten. Echter werd de expressie van SOCS3 enkel verhoogd na stimulatie met recombinant IL-6 en niet door TGF-β, terwijl we eerder hadden aangetoond dat er na TGF-β stimulatie ook IL-6 werd geproduceerd. Deze resultaten suggereren dat TGF-β mogelijk de verdere IL-6 signaalroute remt. Gebaseerd op deze hypothese hebben we onderzocht of TGF-β de effecten van recombinant IL-6 kan remmen. Inderdaad, in aanwezigheid van TGF-β werd de activatie van STAT3 na IL-6 stimulatie sterk geremd. Ook de expressie van STAT3-afhankelijke genen zoals SOCS3, BCL3, MMP1 en SAA1 werd minder sterk geactiveerd door IL-6 als TGF-β aanwezig was. Deze resultaten demonstreren dat TGF-β de signaalroute van IL-6 remt in kraakbeen, ondanks dat er IL-6 wordt geproduceerd door de cellen zelf. Een mogelijk mechanisme hoe TGF-β de IL-6 signaalroute zou kunnen remmen is als het de aanwezigheid van de IL-6 receptor (IL-6R) op celmembranen zou kunnen beïnvloeden. Het bleek inderdaad dat na stimulatie met TGF-β de expressie van de IL-6R in kraakbeen sterk verlaagd was. We hebben dit effect in kraakbeen van meerdere artrose patiënten vastgesteld, wat laat zien dat dit een algemeen mechanisme is. Om te bevestigen dat de verlaging van de IL-6R de onderliggende reden is voor de remming van de IL-6 signaalroute door TGF-β hebben we gebruik gemaakt van een oplosbare variant van de IL-6R (= sIL-6R) die niet aan het membraan van cel gekoppeld zit. Na het toevoegen van sIL-6R, wat de verlaging van de IL-6R op de kraakbeencellen omzeilt, was

het remmende effect van TGF-β op de IL-6 signaalroute verdwenen. Deze resultaten onthullen een nieuwe rol van TGF-β in kraakbeen. In de gewrichtsvloeistof van artrose patiënten is de oplosbare variant van de IL-6R aanwezig, wat mogelijk het beschermende effect van TGF-β omzeilt en bijdraagt aan kraakbeenschade tijdens artrose. Op basis van deze bevindingen zou de oplosbare IL-6R een potentieel therapeutisch target kunnen zijn in artrosepatiënten.

Remming van de IL-6 receptor en signaalroute door TGF-β gaat verloren in verouderd kraakbeen

Nadat we hadden vastgesteld dat TGF-β een beschermende rol heeft in kraakbeen via remmen van de IL-6 signaalroute, vroegen we ons af of dit mechanisme mogelijk beïnvloed wordt door veroudering van het kraakbeen. Een hoge leeftijd is de belangrijkste risicofactor voor artrose. Ons laboratorium heeft reeds aangetoond dat de beschermende TGF-β signaalroute via Smad2/3 minder geactiveerd wordt in verouderd kraakbeen, wat mogelijk het kraakbeen kwetsbaarder maakt voor de ontwikkeling van schade en artrose. In **hoofdstuk vier** hebben we daarom onderzocht of door het verlies van de Smad2/3 signaalroute in verouderd kraakbeen ook de remmende invloed van TGF-β op de IL-6R verloren gaat. Helaas is gezond humaan kraakbeen zeer moeilijk te verkrijgen. Om veroudering in kraakbeen te bestuderen is daarom gebruik gemaakt van runderkraakbeen, aangezien dit sterk lijkt op humaan kraakbeen en beschikbaar is in een brede leeftijdreeks. Alle veranderingen die in verouderd menselijk kraakbeen worden waargenomen zijn ook zichtbaar in verouderd runderkraakbeen, zoals het dunner worden van het kraakbeen, het verlies van kraakbeencellen, en het verlies van kraakbeenbouwstenen zoals aggrecan. Door het gebruik van runderkraakbeen kan bovendien artrotisch kraakbeen worden uitgesloten door middel van visuele inspectie, waardoor artrose en veroudering los van elkaar bestudeerd kunnen worden. Om het effect van veroudering op TGF-β-gemedieerde regulatie van de IL-6 signaalroute te onderzoeken hebben we kraakbeen van het metacarpofalangeale gewricht van 80 koeien verzamelt met een leeftijd van 0.5 -14 jaar oud. We hebben aangetoond dat de genexpressie van de IL-6R sterk toeneemt in verouderd runderkraakbeen. Daarentegen vonden we geen leeftijdsafhankelijke effecten in de genexpressie van IL-6 receptor β (GP130), die belangrijk is voor de signaaltransductie van andere ontstekingsmediatoren van de IL-6 familie zoals oncostatin M en leukemia inhibitory factor. Dit suggereert dat de toename in IL-6R expressie met leeftijd specifiek is voor de IL-6 route. Vervolgens hebben we onderzocht of de verhoogde IL-6R expressie met leeftijd ook resulteert in een verhoogde reactie op IL-6. Inderdaad, na stimulatie met IL-6 werd het intracellulaire signaaleiwit STAT3 sterker geactiveerd met een toenemende leeftijd. Ook de activatie van IL-6 afhankelijke genexpressie die een rol spelen in kraakbeenschade, zoals *bMMP3, bPTGS2* en *bVEGFA*, was sterk verhoogd in verouderd kraakbeen ten opzichte van jong kraakbeen. Deze resultaten bevestigen dat de IL-6R expressie en de IL-6 signaalroute verhoogd zijn in verouderd koeienkraakbeen. Aangezien we in **hoofdstuk drie** hebben laten zien dat TGF-β een belangrijke rol speelt in het remmen van IL-6R expressie, kwamen we tot de hypothese dat verminderde de werking van TGF-β mogelijk de onderliggende

oorzaak is voor de verhoogde IL-6R expressie met leeftijd. Om dit te onderzoeken hebben we runderkraakbeen van koeien van verschillende leeftijden gestimuleerd met TGF-β, en gekeken naar het effect op de expressie van IL-6R. Zoals verwacht was IL-6R expressie sterk verlaagd na TGF-β stimulatie in jong kraakbeen, maar ging dit effect verloren in verouderd kraakbeen. Tevens hebben we onderzocht of het remmend effect van TGF-β op de IL-6 signaalroute verandert in jong ten opzichte van oud koeienkraakbeen. Stimulatie met TGF-β resulteerde in remming van IL-6-gemedieerde activatie van STAT3 en genexpressie van SOCS3 in jong kraakbeen, terwijl in verouderd kraakbeen deze remming van TGF-β sterk verminderd was. Samengenomen laten deze waarnemingen zien dat de verhoging in IL-6R expressie en de IL-6 signaalroute in verouderd kraakbeen verklaard kan worden door verlies van TGF-β-gemedieerde verlaging van de IL-6R, hetgeen in jong kraakbeen nog volop actief is. Aangezien IL-6 een schadelijke rol heeft tijdens artrose ontwikkeling, impliceren deze bevindingen dat het verlies van TGF-β-gemedieerde verlaging van de IL-6R in verouderd kraakbeen zorgt voor verhoogde gevoeligheid voor IL-6 gemedieerde schade, wat mogelijk bijdraagt aan de ontwikkeling van artrose.

De IL-6 signaalroute als therapeutisch doelwit in artrose

In **hoofdstuk drie** en **vier** hebben we een beschermende rol aangetoond voor TGF-β als remmer van de IL-6 signaalroute in kraakbeen, welke mogelijk is verstoord tijdens veroudering of tijdens artrose. Aangezien IL-6 een schadelijke rol heeft in kraakbeen, draagt verstoring van dit beschermende TGF-β mechanisme mogelijk bij aan de ontwikkeling van artrose. Deze bevindingen impliceren dat de IL-6 signaalroute een mogelijk therapeutisch doelwit zou kunnen zijn voor artrose patiënten. De IL-6 signaalroute is uitermate complex, onder andere omdat het op twee verschillende manieren geactiveerd kan worden: via de klassieke en trans-signaalroute. De klassieke route wordt geactiveerd via de IL-6R op het celmembraan, terwijl de trans-signaalroute wordt geactiveerd via de eerdergenoemde oplosbare variant van de IL-6R (sIL-6R). Eerder onderzoek wijst uit dat activatie van de deze routes kan leiden tot andere effecten in gewrichtweefsels, zoals kraakbeen, waarbij de klassieke route vaak beschermend is en de trans-signaalroute juist schadelijk. Aan de hand van literatuuronderzoek, beschrijven we in **hoofdstuk vijf** de verschillende rol van de klassieke en trans-signaalroute van IL-6 in het gewricht en tijdens de ontwikkeling van artrose, en geven we een toekomst perspectief voor therapie ontwikkeling. De onderzochte literatuur wijst uit dat de klassieke IL-6 signaalroute voornamelijk betrokken is bij beschermende effecten binnen het gewricht, zoals in kraakbeen en synovium, door het remmen van kraakbeenafbraak via de productie van beschermende stoffen zoals TIMP-eiwitten. De trans-signaalroute daarentegen, draagt bij aan de productie van kraakbeen afbrekende enzymen, zoals MMP en ADAMTS-eiwitten, en de verhoging van ontsteking in kraakbeen en synovium. Echter plaatsen we ook de kanttekening dat deze scheiding niet altijd zwart-wit is, maar er bijvoorbeeld ook schadelijke effecten worden gevonden van de klassieke IL-6 signaalroute. Mogelijk wordt het uiteindelijke effect van de IL-6 route tevens beïnvloed door het lokale milieu, zoals de aanwezigheid van ontsteking- of groeifactoren. We beschrijven dat lokale regulatie van IL-6R

expressie, bijvoorbeeld door hormonen, cytokines en epigenetische factoren, de functie van IL-6 in het gewricht kan beïnvloeden. Lokale verhoging van de IL-6R, bijvoorbeeld door ontsteking, kan bijdragen aan verhoogde activatie van de klassieke IL-6 signaalroute. Verscheidene *in vivo* studies laten zien dat het remmen van IL-6 tijdens experimentele artrose opgewerkt in muizen een beschermend effect heeft en leidt tot verminderde kraakbeenschade, subchondrale botschade en synoviale ontsteking. Echter maken deze studies geen onderscheid tussen de remming van de klassieke en trans-signaalroute van IL-6. Op basis van de onderzochte literatuur suggereren wij dat dat het specifiek blokkeren van de trans-signaalroute *in vivo* een slimme strategie zou zijn om de beschermende versus de schadelijke effecten van IL-6 te onderscheiden in de toekomst. Voorgaande studies toonden aan dat IL-6 trans-signaalroute geremd kan worden door toevoeging van 'soluble gp130', wat resulteerde in de remming van schadelijke effecten terwijl de beschermende effecten van de klassieke route intact bleven. Wij suggereren dat deze strategie mogelijk ook interessant is voor de behandeling van artrose patiënten, echter is er eerst aanvullend preklinisch onderzoek nodig die de significantie van IL-6 trans signaalroute in muismodellen met experimentele artrose aantoont.

Conclusie

In dit proefschrift beschrijven we interacties tussen de IL-6 en TGF-β signaalroutes in articulair kraakbeen, die mogelijk zeer relevant zijn tijdens kraakbeen homeostase en artroseontwikkeling. We beschrijven een nieuwe, beschermende rol van TGF-β via het remmen van de IL-6 signaalroute door het verlagen van de IL-6R. Tevens laten we zien dat dit beschermende mechanisme mogelijk omzeilt wordt tijdens artrose door de aanwezigheid van soluble IL-6R, of tijdens veroudering door het verlies van TGF-β-gemedieerde IL-6R suppressie. De resultaten gepresenteerd in dit proefschrift bieden een goede basis voor toekomstige fundamentele en klinische studies om te onderzoeken of het remmen van de IL-6 signaalroute een mogelijke therapie kan zijn voor artrose patiënten.

CURRICULUM VITAE

Renske Wiegertjes werd geboren op 28 januari 1993 te Wageningen. Na het behalen van haar VWO-diploma aan het Pantarijn in Wageningen in 2011, ging ze Biomedische Wetenschappen studeren aan de Radboud Universiteit in Nijmegen. Ter afronding van deze bacheloropleiding liep ze in 2013 vier maanden stage op de afdeling Maag-, darm-, leverziekten van Prof. Joost Drenth onder begeleiding van Mark Broekman. Na het behalen van haar bachelor heeft Renske de master Humane Pathobiologie gevolgd aan de Radboud Universiteit Nijmegen. Tijdens haar master heeft Renske deelgenomen aan het interdisciplinaire Radboud Honours Programma: Reflections on Science, waar ze samen met studenten van PennState University een rapport schreef over de toekomst van de gezondheidszorg van ouderen in opdracht van het Nederlandse Ministerie van Volksgezondheid. In het kader van haar master liep Renske verschillende onderzoeksstages, waaronder een stage van 6 maanden aan de universiteit van Kopenhagen in Denemarken in samenwerking met de afdeling Immunologie en Microbiologie en het Copenhagen Center for Glycomics. Tevens heeft zij twee onderzoeksstages op de afdeling Experimentele Reumatologie volbracht onder begeleiding van dr. Edwin Geven en dr. Esmeralda Blaney Davidson. Na het behalen van haar cum-laude master titel in 2016 startte zij binnen diezelfde afdeling als promovendus haar PhD project met als onderwerp ‘Inflammation-induced SOCS3 leads to deleterious chondrocyte behaviour by altering TGF-β signalling, resulting in progressive cartilage damage in osteoarthritis’, waarvan de resultaten beschreven staan in dit proefschrift. Dit onderzoek werd verricht onder begeleiding van Dr. Esmeralda Blaney Davidson en Dr. Fons van de Loo en onder supervisie van Prof. Dr. Peter van der Kraan. Gedurende haar promotieonderzoek heeft Renske op diverse nationale en internationale congressen de mogelijkheden gekregen haar werk te presenteren, waaronder de Nederlandse Vereniging voor Matrix Biologie, Annual meeting of the Osteoarthritis Research Society International (OARSI) en de European Workshop for Rheumatology Research (EWRR). Haar presentatie op de jaarlijkse bijeenkomst van de Nederlandse Vereniging voor Matrix Biologie in 2016 resulteerde in de prijs voor de beste poster presentatie. Sinds september 2020 is Renske werkzaam als Regulatory Affairs Manager bij Synthon in Nijmegen.

LIST OF PUBLICATIONS

- **Wiegertjes R**, Thielen NGM, Mekers VE, van Beuningen HM, van den Akker GGH, Neefjes M, Koenders MI, van Lent PLEM, van der Kraan PM, van de Loo FAJ, Blaney Davidson EN. *Inflammation-induced SOCS3 impairs stem-cell based cartilage formation but does not affect protective TGF-β signaling in articular chondrocytes (in preparation).*

- **Wiegertjes R**, Thielen NGM, van Caam APM, van Laar M, van Beuningen HM, Koenders MI, van Lent PLEM, van der Kraan PM, van de Loo FAJ, Blaney Davidson EN. *Increased IL-6 receptor expression and signaling in ageing cartilage can be explained by loss of TGF-β-mediated IL-6 receptor suppression. (Accepted for publication in Osteoarthritis Cartilage).*

- **Renske Wiegertjes**, Fons A J van de Loo, Esmeralda N Blaney Davidson. *A roadmap to target interleukin-6 in osteoarthritis.* Rheumatology (Oxford) 2020 Oct 1;59(10):2681-2694.

- **R Wiegertjes**, A van Caam, H van Beuningen, M Koenders, P van Lent, P van der Kraan, F van de Loo, E Blaney Davidson. *TGF-β dampens IL-6 signaling in articular chondrocytes by decreasing IL-6 receptor expression*. Osteoarthritis Cartilage 2019 Aug;27(8):1197-1207.

- Mathijs G A Broeren, Claire E J Waterborg, **Renske Wiegertjes**, Rogier M Thurlings , Marije I Koenders, Peter L E M Van Lent, Peter M Van der Kraan, Fons A J Van de Loo. *A three-dimensional model to study human synovial pathology.* ALTEX 2019;36(1):18-28.

- Mark M T J Broekman, Hennie M J Roelofs, Frank Hoentjen, **Renske Wiegertjes**, Nicole Stoel, Leo A Joosten, Dirk J de Jong, Geert J A Wanten. *LPS-Stimulated Whole Blood Cytokine Production Is Not Related to Disease Behavior in Patients with Quiescent Crohn's Disease*. PLoS One 2015 Jul 24;10(7):e0133932.

PHD PORTFOLIO

Name PhD candidate:	**PhD period:**
Renske Wiegertjes	01-09-2016 – 30-06-2020
Department:	***Promotor(s):***
Experimental Rheumatology	Prof. P. van der Kraan
Graduate School:	***Co-promotor(s):***
Radboud Institute for Molecular Life Sciences	Dr. EN Blaney Davidson, Dr. FAJ van de Loo

	Year(s)	ECTS
TRAINING ACTIVITIES		
a) Courses & Workshops		
- Pilot PhD in the Lead designed by PhD students	2016	1.85
- Graduate Course PhD in the Lead	2016	1.0
- Scientific Integrity	2017	1.0
- Management voor promovendi	2017	2.0
- Introductie Nijmeegse Curricula	2017	0.5
- Scientific Writing for PhD students	2018	3.0
- GCP-1 basicursus	2019	0.5
- Solliciteren en Netwerken	2020	1.0
b) Seminars & lectures		
- Radboud Research Rounds lectures	2016-2020	1.2
- Organization LabTours within Research Theme (Inflammatory Diseases) and special edition for PhD students	2018, 2019	0.75
- STAP (Sleutel Tot Actieve PatïentenParticipatie) meeting	2016-2020	1.2
- Weten & Eten Scholingsavond Reumatische Ziekten	2016-2020	0.4
c) Symposia & congresses		
- PhD retreat (Poster presentation)	2016-2019	2.25
- NVMB: Dutch Society of Matrix Biology (Poster presentation)	2016, 2019	1.5
- NVMB: Dutch Society of Matrix Biology (Oral presentation)	2018	0.75
- Osteoarthritis Research Society International (OARSI) World Congress (Poster presentation)	2018	0.75
- European Workshop for Rheumatology Research International congress (Poster presentation)	2019	0.75
d) Other		
- Member of Early Stage Research Committee (ESR) of the NVMB	2018, 2019	2.0
- Co-organizing a 2-day conference (NVMB)	2018, 2019	2.0
- Journal club	2017-2019	1.5
TEACHING ACTIVITIES		
e) Lecturing		
- Teaching Q1 (Verwondering/Aanval en verdediging)	2017, 2018	2.0
- Developing material for MINK07 and teaching	2018, 2019	1.75

f) Supervision of internships / other			
-	Assistance at Stagemarkt Biomedical Sciences	2016-2015	0.5
-	Supervision Bachelor student internship	2017	1.5
-	Supervision Master student internship	2018	2.0
-	Supervision Master student internship	2019	2.0
-	Supervision of 1st year biomedical science students during 'meet the PhD'	2019, 2020	0.75
-	Supervision of high school student for 'snuffelstage'	2019	0.5
TOTAL			**36.9**

RESEARCH DATA MANAGEMENT

Findable

All (raw) data described in this thesis can be found at the department of Experimental Rheumatology of the Radboud university medical center.

Accessible

All (raw) data and protocols in this thesis can be obtained on request from the department of Experimental Rheumatology of the Radboud university medical center.

Interoperable

All (raw) data in this thesis is documented in English according to the FAIR principles and include qualified references to other (meta)data.

Reusable

All (raw) data shown in this thesis are documented to be reusable for future research and processing.

DANKWOORD

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